AN INTRODUCTION TO THE STUDY OF VIRUSES

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BY

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PREFACE

At the time of writing this book, there has been no attempt to present the study of viruses as the separate discipline of—to use a bastard word—virology. Heretofore, the subject has been divided up into artificial compartments, plant viruses, animal viruses, and bacteriophages, as if there were no common bond between them. I have therefore attempted to survey the whole field, superficially no doubt, but in such a way that the relationships between these interesting agents may become clear to the student. In doing this I am aware that I am inviting criticism and that there may be little in common between, say, the viruses of vaccinia or psittacosis and some of the very small plant viruses. We can, however, follow Boycott's suggestion of a descending scale with the smallest organisms at one end and the protein molecule at the other. In between those limits, and perhaps overlapping at each end, lie the viruses, a heterogeneous collection no doubt but all showing some characteristics in common.

Like most modern scientific disciplines, but more so than some, the study of viruses becomes essentially a matter of team work because of its many contacts with other subjects and its wide ramifications through much of the field of natural science. This makes it a difficult matter for one writer to cover the field in an adequate, if only introductory, manner and a severe test of his comprehension of the subject. I have, nevertheless, written this book in the hope that it will be of use, not only to the "virologist," if such a person exists, but also to the biochemist, the biologist, the medical student and anyone else who is interested in that borderland country whose boundaries were described by Aristotle as "indistinct and doubtful."

Grateful acknowledgment is due to my colleague, Dr. Roy Markham, for his constructive criticism of Chapters V to VIII, and for taking many of the photographs used in the illustrations.

K. M. S.

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CHAPTER I

INTRODUCTORY

THERE is little doubt that virus diseases have existed since very early times, though it is not known for certain how early because of the lack of precise descriptions. Evidence has been advanced to show that smallpox existed in China as early as 1700 B.C., and Zinsser (1937) says that it is definite that smallpox was a common condition all through North Africa by the sixth century A.D. By the year 1000 it was present in practically all European nations and was again and again reintroduced from the East by returning crusaders. By the middle of the sixteenth century it is clear that the entire world had become infected with the virus. Of this virus disease Zinsser speaks as follows—

> The smallpox epidemics of the subsequent two centuries, recurring whenever susceptible fuel had accumulated, were of an extent and severity of which it is hard for us to form any conception at the present time; and it is safe to say that this condition would still prevail, attacking each new generation, were it not for the single and simple procedure of Jennerian vaccination.

This statement might well be taken to heart by those responsible for the repeal of the vaccination laws.

The earliest reference to a condition in plants now known to be due to a virus infection is a description published in 1576 by Charles l'Ecluse of a variegation in the colour of tulips, which is now called "breaking" and is due to an aphis-transmitted virus.

Tulips showing this type of "break" are figured in *Theatrum Florae*, published in 1662, and are thought to be the work of the painter Daniel Rabel. About 1670, in *Traité des Tulipes*, the first suggestion was made that the variegation in the flower colour might be due to a disease.

Towards the end of the eighteenth century the "curl" disease of potatoes was much in evidence and the favourite explanation for it was the "degeneration" theory, which supposed the "curl" to be a kind of senile decay caused by long-continued vegetative propagation. We know now that this degeneration is due solely to the infiltration of viruses into the potato stocks and that, if properly protected from

virus infection, potatoes can be propagated indefinitely by vegetative methods.

It was about this time also, 1796 to be exact, that Jenner made his first experiment in vaccination against smallpox by inoculating the arm of a boy named James Phipps from a pustule on the hand of a young woman who had contracted it while milking the cows. Later, the boy was inoculated with the smallpox virus without effect.

In 1886 Mayer described a disease of the tobacco plant which he called Mosaikkrankheit and showed that this "mosaic" disease could be communicated to a healthy tobacco plant by inoculation with the sap of the infected plant. The first scientific demonstration of the existence of a virus was made in 1892 by the Russian botanist, Iwanowsky, who found that the sap from a mosaic-diseased tobacco plant was still infectious after passage of a bacteria-proof filter candle. About six years later, two German workers, Loeffler and Frosch, found that foot-and-mouth disease of cattle was also caused by a filter-passing virus. They made the observation, interesting for that time, that the disease could not be due to a poison because of the infinitesimal quantity which was capable of causing infection.

The next discoveries concerned the mode of spread of certain viruses and showed the relationship existing between viruses and insects. Probably the first to prove experimentally that some relationship existed between viruses and insects was a Japanese farmer, Hashimoto, who worked in 1894 with the dwarf disease of rice and a leaf-hopper. These studies were continued by various Japanese workers and in 1906 the Imperial Agricultural Experiment Station of Japan proved that the leaf-hopper was not itself the cause of the disease but carried to the plant something which caused it.

In America, during the years 1909–15, various workers pointed to some connexion between the curly-top disease of sugar-beet and the leaf-hopper, *Eutettix tenella*. Finally, in 1915, Smith and Boncquet showed that a single insect, after feeding on a diseased sugar-beet, could infect a healthy sugar-beet if allowed to feed on it for five minutes. It was in 1900 that Walter Reed demonstrated that the virus of yellow fever was transmitted by a mosquito.

A chance observation made in 1896 by a bacteriologist, Hankin by name, was to be repeated nearly twenty years later as a discovery of great scientific importance. While making a bacteriological examination of the water of the river Jumna, Hankin found that, whereas immediately below Agra the water contained more than 100,000

bacteria per c.c., only a few miles farther down the river less than a thousandth of that number could be found. Obviously something was destroying the bacteria. In 1915 Twort noticed that clear patches developed in colonies of bacteria growing on an agar plate as if the organisms were being "eaten up." Being interested in "filterable viruses," Twort passed some of the culture showing the clear spots through a bacteria-proof filter candle and added some of the filtrate to a fresh culture of bacteria. He found that the filtrate was capable of producing clear patches in that culture also. This demonstrated that something of a virus nature was destroying the bacteria. A year or two later d'Herelle, who was investigating a bacterial disease of locusts, discovered the same phenomenon and called it the *bacteriophage* (bacterium eater). Now the study of the bacteriophages, or bacterial viruses as they are called at the present time, constitutes an important branch of virus research.

As far back as 1911 the American worker Peyton Rous demonstrated that a malignant new growth in a fowl could be transmitted by means of a cell-free filtrate. This was the first demonstration of the fact that viruses can cause tumours and may yet prove to be a discovery of profound significance. In 1928 Purdy showed that tobacco-mosaic virus was a potent antigen and that when inoculated into a rabbit it gave rise to a specific antiserum. This discovery opened the way to a great deal of intensive work on the serology of plant viruses.

In 1931 Elford developed his new series of graded collodion membranes, called "gradocol" membranes, which have been much used for filtration work with viruses and for measuring their particle size. Beginning at about this period, and from time to time since then, some new plant viruses have been described, the study of which, apart from their intrinsic interest, has yielded results of great scientific importance. Among these viruses may be mentioned that of tomato bushy stunt, the tobacco necrosis viruses, and the virus of turnip yellow mosaic. In 1933 the discovery that influenza was due to a virus was made by Smith, Andrewes, and Laidlaw, and later came the development of the technique for growing this and other viruses on the chorio-allantoic membrane of the developing hen's egg (Burnet, 1935).

In 1935 came the isolation, by Stanley, of the virus of tobacco mosaic in the form of fine needles or paracrystals, an achievement which has stimulated much chemical work on viruses. Shortly after

this, Bawden and Pirie (1937) showed that the virus was a nucleoprotein. They also succeeded in crystallizing the virus of tomato bushy stunt which was the first virus to be crystallized in a three-dimensional form. Other plant viruses which can form true crystals are those of tobacco necrosis, southern bean mosaic, and turnip yellow mosaic. The discovery of crystallizable viruses has enabled X-ray diffraction studies to be made, and photographs taken on the electron microscope of single virus crystals afford information on the molecular arrangement of the crystal faces. The application of electron microscopy to the study of viruses has greatly increased our knowledge of the size and shape of virus particles and may perhaps throw some light on the multiplication mechanism of viruses. The tissue-culture of animal viruses, the egg-membrane technique mentioned above and their passage through unusual animal hosts have led to the production of valuable vaccines, of which that in use against yellow fever is an outstanding example.

As to the exact nature and origin of viruses, various theories have been put forward. One propounded independently by Green (1935) and by Laidlaw (1938) visualizes a virus as a retrograde organism which, having lost the power of making substances essential to its growth, is entirely dependent on the cells of the host for such substances. On this theory the viruses have developed parasitism to its highest possible degree, and in Laidlaw's words, are living a "borrowed life."

Another theory has it that some viruses have no extraneous origin at all but are a product of the cell itself. The virus most quoted in support of this theory is that of potato *paracrinkle* which is present in all plants of the potato variety King Edward but has no known natural means of spread. It can, however, be transmitted to other non-tolerant varieties of potato by grafting and in them it produces a severe disease. Some circumstantial evidence in support of the *de novo* origin of plant viruses is given by the unexplained appearance from time to time of apparently "new" viruses in plants growing under more or less controlled conditions. This question has recently been discussed by van der Plank (1948). The whole question of the heterogenesis, or *de novo* origin, of viruses was debated in a symposium held at Leeds (1946).

CHAPTER II

TRANSMISSION AND SPREAD OF VIRUSES

THE essence of a virus disease is its infectious nature, so that all known viruses are transmissible in one way or another, but the degree of infectiousness differs in the different viruses. It follows, therefore, that the natural mode of spread also differs. In this chapter both natural and artificial means of transmission are considered, but the relationship between viruses and their arthropod vectors is too important to be included here except briefly, and it is dealt with more fully in a subsequent chapter. The various methods of spread, so far as they are known, are dealt with in turn.

Mechanical Contamination, Direct and Indirect

By direct contamination is meant the actual contact between the infected and the healthy organism while indirect contamination involves the spread of the virus by means of infected material, such as saliva, feathers, fomites, etc.

One of the most infectious viruses is that of foot-and-mouth disease, which is spread by direct and indirect contact. The saliva, urine, and milk of infected cattle are all infectious before the appearance of any symptoms, and it is probable that much spread of the disease takes place by indirect contact. When dried on certain materials the virus can remain infective for several weeks; for example, hay treated with infective saliva was capable, a month later, of transmitting the disease to a calf which ate it.

The following incident, recorded by Muessemeier, illustrates the ease of spread of this virus by indirect contact. An outbreak of "bovine pest" in Silesia was isolated by a double ring of military posts which stopped all traffic, human and animal. However, twenty-three days after the establishment of the isolation an outbreak of foot-and-mouth disease developed in the isolated village. Investigation showed that a carter, coming from a village twelve miles away where there was a case of foot-and-mouth disease, had slipped through the military cordon during the night by creeping along a dry ditch, in order to pay a clandestine visit to a girl. He remained at the house a few hours, returning by the same route. The next morning the

girl, going to milk the cows, infected them with the foot-and-mouth disease virus.

There are one or two very infectious virus diseases of fowls in which transmission of the virus can be effected by both direct and indirect contact. *Fowl-pest*, which originated in the Dutch East Indies, is thought to have been brought into England in imported carcasses. The virus can be passed on by direct contact between healthy and infected birds through the medium of the material which is discharged from the beak and nostrils and of the droppings. Infection can also be carried in dirty cages without the infected birds themselves being present. In *fowl-pox* direct contagion is most frequent, but a small wound is apparently essential for infection, such as abrasions of the mouth, which are common as the result of eating grit. Burnet found that virus placed in the drinking water for one month failed to infect pigeons unless some sharp grit was fed at the same time. The manner of transmission of fowl-pox makes an interesting comparison with the spread of certain plant viruses which are considered later.

In *infectious myxomatosis* of domestic rabbits, the tissues, blood, tumour fluids, and nasal secretions are all extremely infectious and it is sufficient to introduce a healthy rabbit into a cage which has contained a diseased rabbit for the former to become infected. In the case of *psittacosis*, or parrot fever, infection has been reported in persons handling imported feathers.

The viruses attacking caterpillars and causing the so-called *polyhedral* and *wilt* diseases are amongst the most infectious. Transmission seems to take place mostly by the mouth. The leaves of the food plant become contaminated either with the polyhedral bodies or with the virus itself, and when the leaves are eaten the virus is ingested at the same time. These viruses are also easily transmitted to caterpillars through a small wound, either artificially or accidentally made. Transmission by mechanical contamination, both direct and indirect, occurs also in some of the plant viruses: *potato virus X* spreads in the field by mechanical contact between infected and healthy potato plants. The virus can only enter the plant through a wound, however small, and this is provided for by the knocking of one plant against its neighbour by the wind and the consequent breaking of leaf hairs or slight abrasions of the leaf surface. *Tobacco necrosis* virus affords an interesting example of the spread of a plant virus by indirect contact; this virus is a peculiar one in many ways and occurs in the

roots of normal-looking plants. The method of spread, so far as is known, appears to be as follows: pieces of dead leaves from infected plants, still containing virus in an infective state, fall on to the surface of the soil in which the virus finally becomes liberated. In the soil it comes into contact with the roots which it enters. We have here an interesting parallel to the infection of pigeons with the fowl-pox virus; it may be remembered that whilst the drinking water containing the virus remained free of grit, infection did not take place and it was only after the addition of sharp pieces of grit that the virus was transmitted. Similarly with the virus of tobacco necrosis, if the plants are grown in water culture containing virus, the roots do not become infected, but if the plants are in soil or sand, the root hairs become damaged during growth movements, and the virus enters through the small wounds thus produced.

There is a virus disease of wheat in the U.S.A., known as *rosette*, which is an apparent example of mechanical contamination, though the exact method of infection is still obscure. Healthy wheat seedlings, transplanted to soil in which diseased wheat plants have been growing, become diseased in their turn. If, however, the soil is sterilized, either by heat or other methods, the plants do not become infected. Soil-inhabiting insects or other arthropods do not appear to be responsible for the spread of the virus and further work is needed on the mechanism of infection in this case.

Transmission by Grafting

In the case of plants, grafting is a certain means of transmitting plant viruses provided organic union between scion and stock is effected. Grafting is the only method by which the *paracrinkle* virus from potatoes of the King Edward variety can be transmitted and is indeed the method by which it was discovered.

Transmission of some plant viruses can also be effected by means of the parasitic plant, *Cuscuta sp.*, and this is really a kind of grafting. By using *Cuscuta* it is often possible to transmit a virus from a host which is unsuitable for study to one which is more convenient. For example the virus of cranberry *false blossom*, which is difficult to study in the cranberry, can be transmitted by means of *Cuscuta* to more suitable plants such as the tobacco or tomato plant. This could be done in no other way since the virus is not sap-transmissible and has a specific insect vector which will not feed upon the tobacco or tomato, and grafting between cranberry and these two plants is not feasible.

Grafting or transplanting is also occasionally used in the study of virus tumours of fowls and ducks, and tumours have been successfully transferred in this way.

Transmission by Inheritance

Hereditary transmission of viruses is not common but there are some authentic cases. Placental transmission to the embryo may occur in certain diseases such as variola, varicella, Rift Valley fever, and rinderpest. Perhaps the most interesting case of hereditary transmission of a virus is that of breast cancer in mice. Some strains of mice may have an incidence of breast cancer in breeding females of 80 to 90 per cent, whilst in others the incidence is almost nil. In hybrids between two such strains it has been found that the incidence of breast cancer in the offspring depends not upon the ordinary laws of inheritance but wholly upon whether the mother came from a high or low cancer stock. In other words the cancer of the breast in specially inbred strains of mice is caused by a virus found in the female mouse's milk. Until very recently it had been assumed that the virus contained in the mother's milk passed into the body of the suckling by way of the alimentary canal. Now, however, experiments suggest that infection takes place through the nose and throat and this is borne out by the inability to detect the virus in the ingested milk in the suckling's stomach.

There is a good deal of evidence which suggests that the polyhedral viruses attacking caterpillars and other insect larvae may occasionally be transmitted through the egg. In an outbreak of polyhedral disease in a stock of caterpillars, there are usually individual larvae which do not succumb to the disease. These larvae complete their metamorphosis but seem to carry the virus with them through the developmental cycle, so that a proportion of the caterpillars of the next generation become infected.

The number of plant viruses which are transmitted through the seed is small and the reasons for the rarity of seed transmission are not known though various conjectures have been put forward, the most likely being the apparent anatomical isolation of the embryo. There are, however, a few authentic cases of the seed transmission of viruses such as those of bean and lettuce mosaic. In this context it should be understood that all plant viruses which are systemic in their hosts are transmitted through vegetative organs such as tubers, rhizomes, cuttings, etc. It is because of this that the virus diseases of

crops like potatoes, strawberries, raspberries, and hops are of such importance.

Air-borne Transmission, including Droplet Infection

It seems likely that air-borne infection does not play a major part in the dissemination of viruses, but this method of transmission may be important in certain cases.

Air-borne infection may be conveyed in large droplet projectiles sprayed short distances from the mouth or nose. Furthermore, similarly expelled droplets 0·1 mm in diameter or less will have become so small through evaporation before they reach the floor that as "droplet nuclei" they can float in the air for many hours or even days. These droplet nuclei may play an important part in the transmission of some viruses. Influenza virus, for example, on reaching the floor, bedclothes, and other objects, may survive on dust particles and these may be subsequently re-suspended in the air. It has been demonstrated that influenza virus of the PR 8 strain could be recovered from dry dust exposed near a ferret infected with influenza. Between 1 and 10 per cent of virus would withstand drying in household dust and the time the virus will remain viable under these conditions has also been demonstrated. Thus there seems to be little depreciation after three days, whilst 10 per cent may persist for a week, and 1 per cent for a fortnight.

Dried epidermal scales from cases of smallpox are regarded as infective and their aerial transfer may carry the infection to a distance. There is also the possibility whether the virus of the common cold might not be shaken out of handkerchiefs and so transmit the infection through the air for short distances. This is a question of considerable importance, and work now in progress suggests that the impregnation of handkerchiefs with a disinfectant might make them less dangerous. Recent experiments have shown that the viruses of poliomyelitis, influenza, and laryngotracheitis can all be transmitted by the exposure of susceptible animals to artificially contaminated air. It has now been shown that the pneumoencephalitis virus of fowls (Newcastle disease or fowl-pest) can be recovered from the air of poultry houses containing infected birds: air from an infected house sampled in 540- or 1080-litre quantities contained virus in sufficient concentration to infect chick embryos. In an attempt to test the infectivity of such air for chickens, four normal chicks were exposed to the aerial environment of a house containing birds affected with pneumoencephalitis.

The four chicks were confined in wire cages suspended $4\frac{1}{2}$ ft above the floor. Care was taken to prevent contact with contaminated material not air-borne. On the sixth day respiratory symptoms were observed in the chicks (Delay *et al.*, 1948). There is probably little doubt that some of the insect viruses causing the polyhedral diseases are also air-borne in the sense that dried polyhedra are capable of being carried in the air for considerable distances. By this means the leaves of the food plant become contaminated and the polyhedra are ingested by the caterpillars.

Arthropod Vectors

The most important of the natural means of transmission of viruses is by agency of insects and, to a lesser degree, of other arthropods, but since the relationship between viruses and their arthropod vectors is an interesting and important one, it is dealt with at some length in the next chapter. For the moment it must suffice to give a list of the more important vectors and the viruses they transmit. Much of the information on the mite and tick vectors is taken from the British Museum (Natural History) pamphlet on *Acari* by S. Finnegan.

TABLE I

VIRUSES TRANSMITTED BY ARTHROPOD VECTORS

Disease	*Vector*	*Host and Possible Vertebrate Reservoir*
Tsutsugamushi disease ("scrub" or "rural" typhus of Far East and "Hill" typhus of India)	Acarina Larvae of: *Trombicula akamuski* (Japan) *T. deliensis* (Malaya) *T. minor* (New Guinea)	Man, bandicoot, field mice
Rocky Mountain spotted fever (Eastern and Western forms)	*Dermacentor andersoni* *D. variabilis*	Man, goats, hares, and small rodents
Fièvre boutonneuse	Dog tick *Rhipicephalus sanguineus*	Man, dog
South African tick typhus	Ticks *Haemaphysalis leachi*	Man, dog
São Paulo rural typhus	Tick *Amblyomma cajennense*	Man, opossum
"Q" fever Australia and U.S.A.	Ticks *Haemaphysalis humerosa* *Dermacentor andersoni* *D. occidentalis* *Amblyomma americanum* *Rhipicephalus sanguineus*	Man, bandicoot
Louping ill	Sheep tick *Ixodes ricinus* L. *Rhipicephalus appendiculatus* Neum.	Sheep

Disease	*Vector*	*Host and Possible Vertebrate Reservoir*
Rickettsial pox	Rodent mite *Allodermanyssus sanguineus*	Man, mouse
Tick-borne fever	*Ixodes ricinus* L.	Sheep
Nairobi sheep disease	*Rhipicephalus appendiculatus* Neum. *Amblyomma variegatum* F.	Sheep
Exanthematic typhus	*Anoplura* Louse *Pediculus humanus*	Man
Trench fever	Louse *P. humanus*	Man
Murine typhus	Rat flea *Xenopsylla cheopis*	Rat (squirrel, shrew)
Yellow fever	Diptera: Mosquitoes *Aëdes aegypti* L. *Aëdes* spp. Stable fly *Stomoxys calcitrans*	Man, monkey, possibly some jungle animals
Dengue	Mosquitoes *Aëdes aegypti* L. *A. albopictus* Skuse	Man
Poliomyelitis (Infantile paralysis)	Possibly muscid flies, house flies, blue-bottles	Man, monkey
Equine encephalomyelitis	Mosquitoes *Aëdes aegypti* *Aëdes* spp. (and also blood-sucking Hemiptera)	Horses, birds, reservoir not certainly known, possibly birds
Rift Valley fever	Mosquitoes *Mansonia fuscopennata* Theo. *Mansonia* spp.	Lambs, cattle, man to a small extent
Blue tongue	Mosquito *Aëdes lineatopennis* Ludl.	Sheep
Sandfly fever	Sandfly *Phlebotomus papatasii* Scop.	Man
African horse sickness	Diptera: Stable Fly *Stomoxys calcitrans*	Horses and mules
Equine infectious anaemia	Stable Fly *S. calcitrans* Horse Fly *Tabanus septentrionalis* Lev.	Horses
Fowl-pox	Mosquitoes of different species and various blood-sucking insects	Fowls, pigeons, turkeys, geese, ducks, guinea fowl, hawks, pheasants, partridges.
		Host
Tomato spotted wilt	Thysanoptera: Thrips *Frankliniella insularis* Frankl. *Thrips tabaci* Lind.	Tomatoes, pineapples, and many ornamental plants
Potato yellow dwarf	Hemiptera: Leaf-hoppers *Agallia constricta* Van Duzee	Potatoes and other solanaceous plants
Aster yellows	*Cicadula sexnotata* Fall.	Aster, and many other plants
Maize streak	*Cicadu'ina mobila* Naude	Maize

		Host
Cranberry false blossom	*Euscelis striatulus* Fall.	Cranberry
Sugar-beet curly-top	*Eutettix tenellus* Baker	Sugar beet, beans, tomatoes, and many other plants
Dwarf disease of rice	*Nephotettix apicalis* Motsch	Rice
Tobacco leaf curl	Aleyrodes: Whiteflies *Bemisia gossypiperda* M. and L.	Tobacco and other plants
Raspberry green and yellow mosaics	Aphididae *Amphorophora rubi* Kalt.	Raspberry
Sugar-beet yellows	*Myzus persicae* Sulz. *Aphis fabae* Scop.	The beet and its varieties, including the wild beet
Cucumber mosaic	*Myzus persicae* Sulz. *Aphis gossipii* Glower *A. fabae* Scop.	Cucurbitaceae and many other plants
Sugar-cane mosaic	*Aphis maidis* Fitch	Sugar cane
Banana bunchy-top	*Pentalonia nigronervosa* Coq.	Bananas
Strawberry crinkle	*Capitophorus fragaefolii* Cockll.	Strawberries
Tulip mosaic or "break"	*Macrosiphum gei* Koch. *Myzus persicae* Sulz.	Tulips and lilies
Potato leaf roll	*Myzus persicae* Sulz. *M. ornatus* Laing *M. convolvuli*	Potato and one or two other solanaceous plants
Potato Virus Y	*M. persicae* Sulz. *Aphis rhamni* Boyer	Potato and other solanaceous plants
Swollen shoot of cocoa	Coccidae Mealybugs *Pseudococcus citri*	Cocoa-tree
Turnip yellow mosaic	Coleoptera: Flea beetles *Phyllotreta undulata* *Phyllotreta* spp. Mustard beetle *Phaedon cochleariae* and several other miscellaneous biting insects	Turnips and some other members of the Cruciferae

Although the list of animal viruses and their arthropod vectors shown in Table I is more or less complete, no attempt has been made to give all the plant viruses and their insect vectors since it would take up a large amount of space and serve no useful purpose. The examples chosen, however, include all the types of insect involved and indicate the marked preponderance as vectors of sap-sucking insects, especially the aphides, over the biting insects.

Miscellaneous Vectors, Intermediate Hosts, and Living Reservoirs of Infection

In the case of the virus of rabies it is necessary for the successful transmission by animals that the saliva or other alimentary secretion containing the virus should be brought into contact with the nerve

tissues of another animal. The virus is then transmitted along a nerve trunk to the central nervous system; the localization of the virus is highly selective, for only nervous tissues are affected. Transmission therefore occurs in nature only by the bite of a mad dog or some other rabid animal. The paralytic form of rabies occurs in South America, frequently in cattle, less frequently in man. In these cases the vector is the vampire bat and the virus has been isolated from the saliva of these animals. The bats apparently affect each other by bites given during quarrels in their roosting places and while a certain number die of the disease, others remain healthy but carry the virus for long periods.

Shope (1941) has carried out some interesting experiments on swine influenza in which he demonstrated that the swine lungworm acts as a reservoir and intermediate host for swine influenza virus. The actual cycle of events seems to be as follows: the developing eggs are deposited by the female lungworm in the lungs of the pig; these are coughed up and swallowed by the pig and eventually passed out with the faeces. The eggs are next swallowed by an earthworm in which they hatch and undergo two further developmental stages. When they reach the third stage, the larvae are ready to infect the pig, and these third-stage larvae become localized in large numbers in the heart, gizzard, and certain glands of the earthworm. Here the larvae must wait until they are swallowed by a pig and they can, if necessary, wait as long as four years in this third-stage larval condition. If the eggs came originally from the lungs of an infected pig, the larvae from these eggs are carrying the influenza virus. The virus, however, is present in the lungworm in a masked, non-infectious form and to induce infection it must be rendered active by the application of a provocative stimulus to the swine it infests. Multiple intramuscular injections of the bacterium, *H. influenzae suis*, furnish a means of provoking infection. Shope found that whilst swine influenza infection can be provoked in properly prepared swine during the autumn, winter, and spring, this could not be achieved in summer. No explanation for this failure has yet been discovered.

Pseudorabies is a very fatal but *non-contagious* disease in cattle and the common laboratory animals, whereas it is a relatively mild but highly contagious disease in swine. The spread of the virus in cattle has been attributed to rats but Shope (1935) has suggested that the part played by rats is not the whole story. He visualizes the epidemiology of the disease as follows: Pseudorabies infection among

wild rats may be maintained in much the same fashion as trichina infection, by cannibalism or by ingestion of virus-containing tissues from other animals. The disease may be transmitted to swine when the carcass of a pseudorabies-infected rat is eaten by a hog. From this hog, the disease spreads to other swine in the same herd as a contagious disease. It is transmitted to swineherds on other farms, either by direct contact of infected pigs with normal pigs, or by the migration of infected rats. With two such efficient means of dissemination of the virus among pigs, one would expect the disease to be prevalent in this species. However, because of the extremely mild nature of porcine pseudorabies, its existence is not suspected. Only when the virus breaks away from the swine reservoir and spreads to cattle is its presence made known. The transmission of the virus from swine to cattle is thought by Shope to take place when the noses of infected pigs come in contact with abraded areas of skin on cattle. It has been shown that in swine the nose serves both for the entrance and the exit of the virus. Furthermore, it has been observed that fatal pseudorabies infection in rabbits can be induced merely by bringing their abraded skin into contact with the noses of infected swine. The blood sera of swine on two farms where pseudorabies had occurred among the cattle were found to be capable of neutralizing pseudorabies virus. It is believed in these instances that the swine had a mild and unrecognized pseudorabies infection and transmitted their disease to the cattle with which they were associated, by transfer of the virus on their noses to the abraded skin of the cattle. The cycle of infection of the pseudorabies virus is thus thought to be as follows: carcasses of cattle dead of the disease, if gnawed by rats, serve as a fresh source of virus from which a rat population can become infected. Pigs become infected through eating the virus-infected carcasses of rats and the infection then spreads through the herd as a mild, highly contagious disease. From the pigs the virus is transmitted to cattle by contact of the noses of infected swine, and so the cycle from rat to swine, to cow, and back to the rat can be completed.

Birds are frequently suspected of being the passive vectors of various animal viruses, more especially of that of foot-and-mouth disease. The situation regarding the starling as a carrier of this virus has been very carefully examined by Bullough (1942) and some of his conclusions are given here. Since the virus of foot-and-mouth disease is capable of retaining infectivity for long periods, two or three months, on such materials as feeding stuffs and bedding, it is theoretically

possible for a bird, whose body was once infected, to retain for some time the power to transmit the disease. The starling is the only British bird which is present in such enormous numbers, which keeps together in such large flocks and which for preference feeds around, or even on, the cattle themselves. The starlings seen in the British Isles are derived from two sources: first the resident birds which are mainly sedentary and secondly the birds of continental origin, coming from Scandinavia and the neighbouring countries, and which spend most of the autumn and winter in this country. The continental birds arrive here in late September, in October, and early November, and leave again in March and early April. Bullough points out one habit of starlings which is of primary importance so far as the transmission of the disease from place to place is concerned. For seven months of the year (June to December) almost all British starlings leave their nesting locality each evening to roost communally on some wood or reed bed and during the whole of the time (October to March) that the continental starlings are present in the British Isles they also travel each day between their feeding grounds and those same communal roosting places. The journeys made are considerable, and a bird often flies a total of forty to fifty miles each day for this purpose alone. During these daily movements the virus of foot-and-mouth disease might be carried to the roost by some infected bird and there passed to other birds which, next morning, might take it away in many other directions. Opportunities for the spread of the virus in the roost at night are very great because large numbers of sleeping birds are packed closely together shoulder to shoulder. At certain seasons of the year, one roost often contains between fifty and a hundred thousand starlings each night. This situation of the possible contamination of other birds in the roosting place offers a parallel to the spread of paralytic rabies among vampire bats in their roosting places.

Bullough has shown that striking correlations exist between the migrations, movements, and distribution of the starlings and the geographical and monthly incidence of the disease. An attempt was also made to analyse the position of foot-and-mouth disease in Finland, Sweden, and Denmark. In the case of Sweden, where the starling is absent in autumn and winter, a graph was obtained which is the reverse of that for the British Isles where, in autumn and winter, the starling is most numerous.

The case thus made for the starling as a transmitter of foot-and-mouth disease virus is fairly strong and would, if substantiated,

explain the spontaneous development of the disease in isolated farms and districts where no other contact with a source of infection can be discovered. The case would, however, be made still stronger if, firstly, it could be demonstrated under experimental conditions that a starling can actually infect a cow by virus carried on its feet or elsewhere, and, secondly, if a starling had ever been found with its body naturally contaminated by virus.

The question of living reservoirs of infection is an important one in the virus field and it is of course intimately bound up with the question of symptomless carriers which is discussed elsewhere. The problem of virus reservoirs has been studied in the cases of jungle yellow fever and of equine encephalomyelitis. In an attempt to find out what jungle animals could act as reservoirs of the yellow fever virus more than two thousand wild animals were captured and inoculated. It was found that a surprising number of these animals were susceptible to the virus and could retain it in the blood for some time without showing any signs of illness. It is tentatively concluded from this that yellow fever is primarily a disease of jungle animals and the transmission of the virus from man to man by the mosquito *Aëdes aegypti* is in the nature of a secondary cycle.

The reservoirs of infection of equine encephalomyelitis virus do not seem to have been identified, though certain birds such as egrets have been suspected of acting as carriers of this virus.

Viruses Which Spread by Unknown Means or Which do not Spread at All

Among the plant viruses are several which spread by unknown methods; in some cases this lack of knowledge may be due merely to insufficient investigation of the problem and no doubt the mode of spread of all these viruses will be elucidated in time. However, in other cases, in spite of careful investigation the problem has not been solved. Attempts to find the insect vectors of tomato bushy stunt virus, of tomato black ring virus, and of wheat rosette virus, to quote some examples, have failed and yet there seems no doubt that these three viruses do spread in nature. The same applies to the viruses of lovage mosaic, *Arabis* mosaic and broken ringspot, but since these viruses have each been isolated from a single plant we have no evidence that they can be spread from plant to plant other than by artificial means.

The best known example of a virus which, so far as we know, does not spread at all, is that of the paracrinkle disease of the potato plant (Salaman and Le Pelley, 1930). This virus, which is present in all plants of the King Edward variety, has never been found in nature in any other potato variety or plant and can only be transmitted by means of grafting. It is indeed quoted as evidence for the heterogenesis —or spontaneous generation—of viruses by those who support this hypothesis. Similarly nothing seems to be known of the method of spread of the Rous sarcoma virus or if it spreads at all.

"Lighting-up" of Latent or "Silent" Viruses

We have seen already a good example of the "lighting-up" of a latent virus when discussing swine influenza. It will be recalled that the virus is latent in its intermediate host, the swine lungworm, and to induce infection in the pig it must be rendered active by the application of a provocative stimulus. Multiple intramuscular injection of the bacterium *H. influenzae suis* furnishes a means of provoking infection.

Serial passage of one virus through successive mice sometimes brings to light another virus, different from the one under study, which has apparently been stimulated into action by the progressive passages.

It has been suggested that some such process takes place in the production of tumours with provocative carcinogens such as the tarring of the skin in rabbits and chickens. For example, Rous (1943) suggests that the carcinogens have, in common, an ability to play upon the tumour-forming potentialities of the cells, with the result that these become realities. The potentialities themselves may be viruses incapable of causing growths unless the local conditions happen to be right or are made right, and even then perhaps only as the result of virus variation.

There is a good deal of evidence that the polyhedral viruses affecting caterpillars can remain in a latent condition for several generations. Then, when circumstances are favourable, the virus starts to multiply and the disease breaks out in epidemic form.

With some latent plant viruses, an apparent exacerbation of the disease caused can be induced by progressive passage of the virus through susceptible plants. In these cases, however, the increase in virulence is more apparent than real and is due more to a selective action than to a stimulation of the virus. A case in point is the

continued passage of potato virus X through successive tobacco plants. This frequently results in an increase in the virulence of the disease. Since, however, this virus commonly occurs in mixtures of strains, differing in virulence, it is probable that during the progressive passages a more virulent strain is selected out.

It sometimes happens that passage of potato virus X through, or propagation in, an unusual plant host results in the production either of a more severe or a much milder virus when returned to the original host. This type of phenomenon can be explained on the grounds that the unusual host is either more or less favourable to the multiplication of the various strains.

CHAPTER III

ARTHROPOD RELATIONSHIPS WITH VIRUSES

THE relationship of viruses with the insects and other arthropods which transmit them is an interesting and important subject, and one which is far from being clearly understood. We do, however, know a great many facts about this relationship and they are discussed, under various headings, in this chapter.

Whereas the number of plant viruses which depend upon insects for their dissemination in the field is much greater than is the case with animal viruses, it seems that the relationship between virus and insect is closer with animal than with plant viruses. This is shown by the apparent multiplication of certain animal viruses in their insect vectors, evidence for which is more easily obtainable and more convincing than is the case with the insect vectors of plant viruses.

Different Types of Arthropod Vectors

As would be expected, the great majority of insect vectors transmit the virus which they carry to the susceptible host during the process of feeding upon that host. There are, however, one or two exceptions to this general rule, such as the apparently mechanical transmission of poliomyelitis virus by non-blood-sucking flies and the possible transmission of trench fever in the excrement of lice rather than by their blood-sucking habits. Occasional spread of the virus of turnip yellow mosaic by means of the faeces of the insect vector may also occur, since passage of the alimentary canal of the insect seems to have little effect on the infectivity of the virus.

The sucking insect, whether its food be blood or sap, is obviously well adapted for transmitting viruses, which are mostly injected into the host together with the saliva. In the case of the animal viruses, the arthropod vectors are, of course, all blood suckers, and although there are differences in the mouth parts, those of the mosquito are perhaps the most typical. Insects which feed on plants, however, can be divided into two categories: *biting insects* such as caterpillars and beetles, which actually eat the leaves and ingest the tissue, and *sucking insects* such as aphides, leaf-hoppers, whiteflies, etc., which feed only on the sap. The vast majority of the insect vectors of plant

viruses fall into the latter category, although there are one or two authentic instances of plant virus transmission by biting insects.

The mechanics of inoculation by a sap-sucking insect are briefly as follows: there are two pairs of very fine stylets, mandibles, and maxillae, interlocking on their inner faces, which fit closely together. Two pairs of grooves in the faces of the stylets thus come together and form two channels up one of which flows the sap of the plant, while the saliva flows down the other. These stylets are contained in an outer beak or rostrum which acts as a supporting structure for the stylets and does not enter the plant at all. When the insect presses on the leaf surface with its beak (labium), the latter bends and becomes foreshortened, whilst the stylets enter the plant. The fact that the labium has this dorsal opening allows the stylets to become free over part of their length during the process of feeding (Plate I, upper photograph).

In the case of one plant virus, that of tomato spotted wilt, the vector, a thrips, has slightly different mouth parts. They consist of a hollow cone, in which work the maxillae and one mandible, the other being vestigial and non-functional. The feeding motion consists of a kind of pickaxe movement with the mandible which rasps off the epidermis, the sap being sucked up as it is liberated. In this insect, therefore, there is not the deep penetration of the plant tissue during feeding which is characteristic of the feeding of such insects as aphides and leaf-hoppers.

Finally we have the biting insects such as the mustard beetle (*Phaedon cochleariae*) and its larvae and the flea beetles, *Phyllotreta spp.*, which transmit the virus of turnip yellow mosaic. Here the mouth parts are entirely different and consist essentially of a pair of strong, chitinized jaws working horizontally in a scissor-like action. The mechanics of infection with this type of insect are thought to be of two kinds, first a direct transmission of virus which contaminates the mandibles; this is a rather rare method of infection. The second kind is a somewhat delayed form of transmission and is thought to be brought about through regurgitation of part of the contents of the foregut during feeding, whereby some of the virus already ingested comes into contact with the leaves of healthy plants.

The rickettsial diseases of man are transmitted by four groups of parasites, ticks, mites, lice, and fleas.

Diseases of the spotted fever and boutonneuse fever groups are transmitted by ticks and it is thought highly possible that ticks are also concerned in the transmission of "Q" fever. Mites have been proved

to transmit only one rickettsial disease, scrub typhus or tsutsugamushi disease. Transmission of endemic typhus by mites has been demonstrated experimentally.

The vectors of epidemic or European typhus and of endemic or murine typhus are lice and fleas respectively (Kohls, 1947).

Specificity of Vectors for Viruses and Selective Transmission

The view was largely held at one time that many insect-transmitted viruses could be spread only by one species of insect. Now it is realized that this species-specificity is rather rare and that a group-specificity is more usual. For example, there are several plant viruses which are spread by more than one species of aphis and several which are spread by more than one species of leaf-hopper but, so far as we know, no aphis-borne virus can be transmitted by a leaf-hopper, nor can a virus which is transmitted by a leaf-hopper be carried by an aphis. There is one case, however, in which a virus transmitted by a beetle can also be carried by an aphis. The virus in question is that of squash mosaic (Freitag, 1941).

This group-specificity of arthropod vectors also exists with animal viruses but does seem to break down in some cases. The virus of dengue appears to be carried only by mosquitoes but that of equine encephalomyelitis, another mosquito-borne virus, has been found in a tick and in a blood-sucking bug (Hemiptera).

Again, the virus of yellow fever can be transmitted by fourteen different species of mosquito and also, according to the literature, by quite a different type of blood-sucking fly belonging to the Muscidae. In addition there are records of the yellow fever virus being carried by blood-sucking bugs (Hemiptera) and by ticks (Acarina). These last cases are probably examples of mechanical transmission and may be purely incidental.

Among the arthropod vectors of animal viruses, the two most important groups are the Culicine mosquitoes and the ticks. Findlay (1936) points out the interesting fact that out of the great mosquito family Culicidae, the tribe *Culicini* alone transmits viruses, the equally numerous tribe *Anophelini* being apparently unable to do so. On the other hand, the *Anophelini* comprise all the carriers of malarial protozoa with the exception of certain species of the genus *Culex* which transmit the organism of bird malaria.

Examples of the species-specificity referred to above, where only one species of insect can transmit a virus, are found in the insect

transmission of two plant viruses, those of potato yellow dwarf and sugar-beet curly-top respectively. These two viruses are transmitted by the leaf hoppers, *Agallia constricta* and *Eutettix tenella*. This extreme specificity of vector is carried a stage farther by the fact that there exist two strains of the potato yellow dwarf and sugar-beet curly-top viruses each with its own vector. Thus the New Jersey strain of potato yellow dwarf is transmitted by *Agallia constricta*, and the New York strain by *Aceratagallia sanguinolenta*. Apparently this specificity is absolute, and neither insect can transmit the other virus. Similarly the strain of beet curly-top in the U.S.A. is transmitted by the leaf-hopper, *Eutettix tenella*, but this insect cannot spread the beet curly-top in Argentina which has the leaf-hopper, *Agalliana ensigera*, as its specific vector. An analogous case exists with the virus of equine encephalomyelitis which occurs in two distinct strains, the Eastern and the Western. The vector of the Western strain is the mosquito *Aëdes aegypti* which cannot normally transmit the other strain.

Selective Transmission of Viruses from a Complex

It is no unusual thing for a plant virus disease to be caused by two or more viruses acting together, and the insect transmission from such composite diseases frequently shows some curious anomalies; the following instances illustrate this and refer only to aphis-borne viruses—

(1) Two viruses present but only one aphis-transmitted.

(2) Two aphis-borne viruses present but they differ in their relationship to different aphis species; thus one species of aphis will pick up both viruses whilst another will pick up only one.

(3) Separation of a virus complex by differential feeding times of the aphis vectors.

The following examples may make these three types of selective transmission clearer. There is a common crinkle disease occurring in the potato plant caused by two viruses acting in unison. These two viruses are known as potato viruses X and Y, and whilst both viruses are sap-transmissible only one, potato virus Y, is aphis-borne. Therefore, whilst sap-inoculation reproduces the whole disease, aphis transmission results in the production of a disease caused by the Y virus alone.

In the second instance, the component viruses are both aphis-borne but differ in their relationships with different aphis species. The two viruses of cabbage black ringspot and cauliflower mosaic often occur

together in the same plant, and if this plant is colonized by the common cabbage aphis (*Brevicoryne brassicae*), or the potato aphis (*Myzus persicae*) both viruses are transmitted. If, however, the plant is colonized by *Myzus ornatus*, only one virus is picked up since this particular aphis does not transmit the second virus of the complex (Kvicala, 1945).

In the third instance, selective transmission is brought about by varying the length of time the aphis is allowed to feed on the plant, in this case a strawberry plant, containing the virus complex. Some viruses are best transmitted if the aphis feeds for five minutes only, whilst others require a minimum time of feeding of some hours for transmission to take place. Such viruses are known as non-persistent and persistent viruses respectively and this phenomenon is more fully explained in a later paragraph.

A fourth anomaly arising from the aphis transmission of virus complexes may appropriately be considered here, although it is not a question of selective transmission by the insect itself. It sometimes, though rarely, happens with a virus complex that one of the component viruses is only aphis-transmitted if the other virus is present with it in the plant. The best-known example of this phenomenon is tobacco rosette, the constituent viruses of which are the mottle and vein-distorting viruses respectively, and it is the mottle virus which is not aphis-borne unless the vein-distorting virus is present with it in the plant (Smith, 1946).

Multiplication of Viruses in their Vectors

The evidence which suggests that viruses may multiply inside the bodies of insect vectors is stronger in the case of the animal viruses than in that of the plant viruses. It has been shown by Merrill and Ten Broeck (1934) that the virus of yellow fever multiplies inside the body of the mosquito vector and Trager (1938) has actually cultivated the virus of equine encephalomyelitis in the tissues of the mosquito.

It would perhaps be more interesting if it could be proved that plant viruses multiply in their insect vectors because that would relate them more closely to the animal viruses. There is a certain amount of rather circumstantial evidence which supports the multiplication theory. For example Black (1941) carried out the following experiment with the aster yellows virus and its leaf-hopper vector (*Cicadula sexnotata*). He selected a number of insects uniform in size, age, and sex, and fed them for a given period on a yellowed aster plant. He

assumed that all the insects received approximately the same dose of virus. After feeding on the aster they were transferred to rye seedlings which are immune to the aster yellows virus. Thereafter the insects were removed in batches at regular intervals and macerated with water to form a paste. This was inoculated at various dilutions into the bodies of known virus-free leaf-hoppers, and the leaf-hoppers were colonized on healthy aster seedlings. Such a procedure is necessary because the aster yellows virus cannot be transmitted mechanically by sap-inoculation and the technique is based on the earlier work of Storey (1932) with the streak virus of maize. Black found that the longer the leaf-hoppers remained on the rye seedlings before being ground up and diluted, the more they could be diluted whilst still being infective when injected into the transmitting leaf-hopper. Insects macerated before the fourth day gave no infection, whereas those macerated on the twelfth day gave infection when diluted 1 : 1000. Black interprets this as evidence that the virus multiplied in the insects when colonized on the rye plant; but Bawden (1943) has criticized this interpretation on the ground that the number of successful inoculations is usually greater if the extracts of macerated insects are diluted 1 : 1000 than if diluted 1 : 100 or 1 : 10. He suggests that this is due to the presence of an inhibitor (Black, 1939) which becomes less effective with dilution. This criticism would be valid enough if the macerated insects were being *inoculated* to the test plant, for only under these conditions does the inhibitor come into action, but since they are inoculated into a virus-free insect which then infects the plant, the presence of the inhibitor is immaterial and the quantity of virus present is the vital factor.

Kunkel (1937) has carried out experiments on the same leaf-hopper and virus and has also obtained results which he considers support the theory of virus multiplication in the insect. He exposed infective insects to high temperatures for varying periods and noted the effect of this on the insect's power to transmit the virus. After exposure for one day to a temperature of 32°C, the leaf-hoppers lost the power to infect healthy plants but quickly regained infectivity, without recourse to a fresh source of virus, when the temperature was lowered. After exposure for a week to high temperatures, the power to infect was regained but much more slowly, whilst insects exposed for twelve days failed to regain it at all. Kunkel interprets these results as indicating a reduction by heat of the virus content of the insect's body below the minimum dose necessary for infection. He considers that

the time required for the treated insects to regain infective power is a latent period while the virus is multiplying up inside them to the necessary concentration for infection and explains the inability of the insects, after being heated for twelve days, to regain infective power at all on the assumption that all the virus has been destroyed. This explanation may be the correct one but it seems somewhat facile, since we do not really know what the effect of heat on virus and insect may be. It is possible, for instance, that the virus is destroyed in one part of the insect only and the time lag is due to the necessity of more virus moving to take its place.

On the other hand it is quite clear that many plant viruses do not multiply inside their insect vectors and indeed are rapidly lost, probably by the action of digestive enzymes. As regards certain viruses which are retained for long periods by their vectors, that of sugar-beet curly-top for example, there is some evidence that the prolonged infectivity is due to a storage, rather than multiplication, of virus. This is suggested by the fact that there seems to be some correlation between length of time of feeding on a source of virus and the length of time the virus is retained by the insect. Again, if there *is* multiplication of the curly-top virus in the leaf-hopper vector, it is not sufficient to keep the virus content of the insect at infection point, since in a series of daily transfers to healthy plants the power to infect generally falls off.

Mechanical Transmission of Virus by Insects

There is probably no sharp line of demarcation between a purely mechanical transmission of viruses by insects and transmission by specific insects only, for between these two extremes can be found intermediate relationships. Presumably the least mechanical form of transmission is found in those insects which retain virus for very long periods, or in those where multiplication is known to take place, or where it can be passed on to the progeny. It has been suggested that rickettsiae were originally insect parasites and certainly the louse is doomed to die from the typhus rickettsiae more certainly than the human being it may infect. But this seems to be the only instance known where an insect vector is adversely affected by the virus it carries.

Possibly a truly mechanical vector would be one which is not parasitic on the organism it infects, as, for example, house flies and other non-blood-sucking muscid flies which are said to transmit the virus

of poliomyelitis and which presumably ingest the virus from contaminated faeces and thus spread it around on food, etc.

In those cases where a virus is spread by a large number of miscellaneous insects and other arthropods it is legitimate to suppose that the transmission is purely mechanical. The virus of fowl-pox for instance seems to be carried by any blood-sucking arthropod which happens to feed on an infected bird; it apparently adheres to the outside of the mouthparts and is said not to enter the body of the mosquito. Since the fowl-pox virus is so infectious, a needle stuck first into an infected bird and then into a healthy one might serve the same purpose.

In the case of turnip yellow mosaic virus which is spread by many kinds of biting insects we have a possible instance of two kinds of mechanical transmission. There is the less usual method where virus, contaminating the jaws, is transferred directly to the plant tissues; more commonly, however, infection apparently takes place from virus which has been ingested and is continually being regurgitated from the foregut during the feeding process. There is the third possibility of plants being infected from the faeces of insects which have fed on infected plants, since the virus passes through the body without apparent loss of infectivity.

It would be legitimate to postulate that all insect-transmitted plant viruses are transmitted mechanically except in those cases where it may in the future be proved that the virus multiplies in the insect. The extreme specificity of insect transmission of some viruses could be explained on the presence or absence of certain digestive enzymes, *p*H suitability, location of feeding and so forth without there being a fundamental relationship between insect and virus which involves multiplication. On the other hand, even multiplication of a virus in its insect vector does not necessitate extreme specificity. Both the yellow fever virus and that of equine encephalomyelitis are said to multiply in their mosquito vectors and yet the former is spread by many kinds of mosquito and the latter apparently by such different types of arthropod as mosquitoes, blood-sucking bugs, and possibly ticks.

Retention of Viruses by their Vectors: Persistent and Non-persistent Viruses

In the case of such animal viruses as those of yellow fever and equine encephalomyelitis where there seems to be a biological

relationship between virus and insect, in that the virus can multiply inside the vector, it is perhaps to be expected that the insect should retain the virus for long periods, frequently for the rest of its life without recourse to a fresh source of virus. In the case of a virus like that of fowl-pox which may be carried mechanically on the insect's mouth-parts, it is reasonable to suppose that infectivity is rapidly lost by the insect.

So far as the plant viruses are concerned, we can divide those which are insect-transmitted into two classes, although there is no hard and fast dividing line between them. In a series of progressive 24-hour transfers of infected insects from plant to plant, there is one class of viruses which is not carried beyond the first plant and is thus rapidly lost by its insect vector. This type of virus is mostly aphis-transmitted and has been named *non-persistent* (Watson and Roberts, 1939). In a similar series of 24-hour transfers, the second type of virus, called *persistent*, is not as a rule transmitted to the first plant of the series, but is transmitted to the succeeding plants and is usually retained by the insect for long periods if not for the rest of its life. Intermediate between these two types is the virus of turnip yellow mosaic previously mentioned which seems to be partly non-persistent and partly persistent. Occasionally the virus is transmitted in what seems to be a purely mechanical method, by adhering to the mouthparts of the beetle vector; this might be termed non-persistent. On the other hand, the vector can retain the virus for several days and continue to infect plants in progressive series; this is the persistent phase and can be accounted for by regurgitation at intervals of infected material from the foregut of the insect during mastication of the leaf.

When the virus passes into the body of the insect vector and is rapidly lost it is reasonable to suppose that digestive enzymes in the insect's body inactivate the virus. This supposition is supported by the fact that continuous feeding of the aphis vector, whether on a healthy or infected plant, greatly reduces the efficiency of the vector, whilst a period of starving before feeding on a source of virus followed by an extremely short—about two minutes—feed on the healthy plant are the optimum conditions for infection. Starving the insect after feeding on the source of virus also prolongs the period during which the aphis is infective. All these facts support the suggestion that non-persistent viruses are rapidly digested in the insect's body; starving both before and after feeding would presumably reduce the flow of digestive enzymes whereas long periods of feeding, either

on the diseased or healthy plant, might be expected to increase the flow of enzymes and so reduce the insect's efficiency as a vector.

Effect of Viruses on their Vectors

There is very little evidence to suggest that an arthropod vector of viruses is in any way affected by the virus it may transmit. Even when there is good evidence that the virus multiplies inside the body, as is the case with certain mosquito-borne viruses, the insects do not seem to be adversely affected.

The only case known of an arthropod vector being affected by the virus it transmits is that of the louse (*Pediculus*) which is itself killed by the rickettsiae of typhus fever. But the suggestion has been made that typhus fever, like other rickettsial diseases, was originally a disease of insects which has become adapted to the human host.

Transmission of Viruses to the Progeny of Infective Vectors

The inheritance of a virus by the progeny of infective vectors is a rare occurrence and there are only two authentic records in the plant viruses. In the animal viruses there are one or two instances of infection passing through the eggs of certain mites and ticks, and in some cases the larvae of a tick can pass on infection to the nymphae, and freshly infected nymphae can transmit infection to the adults.

Fukushi (1939) presents data showing that the virus of the dwarf disease of rice is passed from parent to offspring to the third generation without recourse to a fresh supply of virus. For the virus to be inherited it is necessary for the female parent to be infective; no virus was passed on to the eggs when the male parent only was virus-carrying.

Recently another case of virus-inheritance has been described: Black (1948) gives evidence of the transmission of the clover club-leaf virus through the eggs of its insect vector. Twenty-four out of twenty-seven insects arising from eggs laid by an infective (i.e. viruliferous) female, transmitted the disease (89 per cent). None of the insects transmitted the virus until at least three weeks after hatching. The best transmissions occurred during the seventh to eleventh weeks inclusive.

As regards the inheritance of infection by vectors of animal viruses, this seems to be confined to rickettsial diseases of which the vectors are mostly mites and ticks.

The life history of the trombiculid mites, as pointed out by Kohls (1947), is of special interest in relation to disease transmission since

only the larvae are parasitic on animals. So far as is known, the eggs are deposited in the soil; the almost microscopic larvae feed on vertebrate hosts of different kinds, become fully engorged and drop to the ground. The nymphs and adults live in the soil, but little is known of their feeding habits. Since the larval mites do not move from host to host, it is clear that the disease agent must pass from parent to offspring for any transmission to occur. This has been proved experimentally when laboratory-reared larvae from female mites collected in the field produced scrub typhus infection in a white rat on which they were fed. Another example of virus inheritance by mites is shown by the virus of St. Louis encephalitis which is transmitted through the eggs of the chicken mite *Dermanyssus gallinae*. Syverton and Berry (1941) have demonstrated that the wood tick, *Dermacentor andersoni* Stiles, can acquire the virus of the western type of equine encephalomyelitis, pass it to later stages in its life-cycle and to its progeny and transmit it. Ticks of the genus *Dermacentor* have essentially the same seasonal occurrence and geographical distribution as the equine encephalomyelitis virus and their habits make them almost ideal vectors or reservoirs of the virus.

The tick-transmission of viruses may be rather complicated; it has been shown by Daubney and Hudson (1931) that infection with Nairobi sheep disease virus of any instar of *Rhipicephalus appendiculatus* results in transmission of the virus by the succeeding stage which then loses infection at the next moult. A female tick, infected as an adult, thus only passes infection to the eggs.

Nature of Relationship between Viruses and Vectors

It has already been suggested in this chapter that, so far as the insect-transmitted plant viruses are concerned, it may be legitimate to regard them all as examples of mechanical transmission in the sense that there is no direct biological relationship between virus and vector, such as a cycle of development inside the insect. What happens, then, to the virus which is swallowed by the insect? In the case of persistent viruses with sap-sucking insect vectors presumably something of this sort takes place. After the virus has been swallowed by the aphis, it cannot be regurgitated, since there is an oesophageal valve which prevents this, but must travel on down the alimentary canal (Plate I). Now in the case of a sap-sucking insect, the only way a virus can reach the plant again is via the saliva and in order to reach the salivary glands it must presumably pass through the wall of the alimentary canal into

the blood. Some evidence for this sequence of happenings is given by the work of Storey (1932). Working with the streak disease of maize which has a specific leaf-hopper vector, he found that these insects could be divided into two races, one which could transmit the virus, termed *active*, and one which could not, termed *inactive*. Both races appeared identical and were undoubtedly only one species. Now both the active and inactive races of this leaf-hopper ingest the maize streak virus and pass it out with the faeces where its presence can be ascertained, but the virus is present in the blood only of the active transmitting race. If, however, a puncture is made with a sterile needle in the inactive insect's abdomen in such a way as to rupture the abdomen, the insect will then behave like the active type and can transmit the virus. The physiological basis of inactivity thus lies in some property of the intestine that prevents the outward movement of virus through its wall. Storey (1939) goes farther and suggests that in this insect the protoplasm of the cells forming the intestinal wall inherits a susceptibility or resistance to invasion by the virus.

A very interesting parallel to this phenomenon is found in an animal virus, that of equine encephalomyelitis and its insect vector, the mosquito *Aëdes aegypti*. It will be remembered that there seems to be a definite relationship between these two since the virus is known to multiply inside the mosquito (Merrill and Ten Broeck, 1934). Two strains of this virus exist, the "eastern" and the "western," of which only the western strain is normally transmissible by the mosquito. Merrill and Ten Broeck showed (1935) that the mosquito could be induced to transmit the eastern strain if it received virus by inoculation into the abdomen, or if it were punctured in the abdomen after a virus feed. In this case it appears as if it was the virus itself which determined activity or inactivity and suggests a closer biological relationship between the virus and the insect's protoplasm than seems to exist in the case of the maize streak virus and its insect vector.

For the transmission of a plant virus by a sap-sucking insect, it is therefore a fundamental necessity that the virus must pass through the wall of the alimentary canal and enter the blood if it is to reach the exterior by way of the saliva. But for the transmission of the turnip yellow mosaic virus by beetles and some other biting insects, this condition does not apply. We know that the virus passes down the alimentary canal and can be recovered, still highly infective, in the faeces but we do not yet know if it reaches the blood. However, this

fact is no longer significant for virus transmission because the beetle vectors and their larvae lack salivary glands, and the mechanism of transmission, as previously mentioned, is probably by regurgitation of infected material from the crop. This supposition is further strengthened by the apparent inability of lepidopterous larvae, which do not regurgitate, to transmit the virus.

When non-viruliferous leaf-hoppers are inoculated in the abdomen with the virus of which they are the natural vectors, they become viruliferous and transmit the virus because the virus enters the blood and later the saliva. But when the beetle larvae vectors of turnip yellow mosaic virus are inoculated in the abdomen, they do not become viruliferous. In this case presumably the virus simply passes outside the body with the faeces; it cannot pass upwards into the crop and be regurgitated because of the valve situated at the posterior end of the crop (Markham and Smith, 1949).

CHAPTER IV

SOME REPRESENTATIVE VIRUS DISEASES

In this chapter short descriptions are given of a number of virus diseases of plants and animals. They are chosen as being as representative as possible of the wide variety of symptoms which infection with viruses calls forth.

In plants, the first virus disease to be described was tobacco *mosaic* and the name was given because of the fancied resemblance of the leaf-mottling to a mosaic pattern. This term is now used to describe a large number of virus diseases where the main characteristic is a mottling of the leaves (Plate II).

In tobacco plants infected with the tobacco mosaic virus, the first sign of infection is an intensification or "picking out" of the veins of the youngest leaves; this phenomenon, known as "clearing of the veins," is a common initial symptom of many mosaic diseases. After a few days the vein-clearing disappears and a mottling of the leaves develops; the mottling varies somewhat in appearance according to environmental conditions but consists usually of dark green areas, sometimes in the neighbourhood of the vein, upon a lighter green background. Frequently large blisters of green tissue are raised or sunken yellow areas may develop together with a certain amount of malformation of the leaves.

Included in the general category of mosaic diseases is a different type of response to virus infection which is usually known as a *ringspot* disease. There are various types of ringspot patterns; the commonest consists of numbers of concentric rings with a spot in the centre (Plate III). Sometimes the rings are single, at other times three or four concentric rings occur at a time; in some diseases the number may reach six or seven. The type of ring may differ also, but not as a rule in the same disease. The rings may be narrow and indented; these are necrotic and cause the death of the cells. Alternatively, rings of a lighter green colour, chlorotic, upon a darker background, may develop. There is no central spot to this type of ring.

There are variations in the symptoms of ringspot diseases. Sometimes instead of rings, complicated ring and line, or oakleaf, patterns may develop in the leaves. There is no very convincing explanation

for this curious development of concentric rings on the leaves of infected plants, though various explanations have been put forward. Whatever the explanation, the phenomenon itself is common enough and there are at least a dozen unrelated viruses which give rise to this type of symptom. In one or two cases, the rings are not confined to the leaves but develop also on the flower petals and even on the fruit.

In both mosaic and ringspot diseases there is often an initial reaction on the inoculated leaf or the site of infection. This takes the form of *local lesions* which may be either rings or solid spots of dead cells. An internal symptom is also associated with these diseases and is known as an "intracellular inclusion." It consists of a rounded mass of cytoplasm, frequently vacuolated and in close association with the nucleus.

Various types of *distortion* are common in plant virus diseases and may be accompanied by mottling in some cases. There is, for example, a strain of the tobacco mosaic virus which distorts the leaves instead of mottling them. Other viruses give rise to leaf rolling, suppression of the leaf blade, distortion of the veins (Plate IV), and the production of abnormal or false blossoms.

As we shall see later in describing some animal virus diseases, *tumours*, and outgrowths of tissue are characteristic of one or two virus infections.

The commonest type of virus outgrowth in plants is known as an *enation* and consists essentially of a new leaflet growing out from the under surface of the normal leaf. Palisade tissue is formed in the veins and this proliferates to form the new leaflets.

Since these enations are organized growth, they cannot be compared with the true virus tumours or cancers which occur in animals. There is, however, at least one apparently true virus tumour in plants. This is caused by the *wound tumour virus* and affects plants of the leguminosae (Plate V). This unusual virus was described by Black (1945, 1946, 1947), who suggests that the following facts are of particular importance when comparing this plant virus tumour with the formation of tumours generally. No causal micro-organism can be detected in the tumour tissue. The disease is not contagious and requires the intervention of a specific insect vector for its spread. Apparently several factors may play a vital secondary role in starting tumour growth in plants invaded by the virus. Wounding is one of these factors; heredity of the host plant is another, whilst the age of the wounded tissue also appears to be important. The tumour tissue never reverts to

normal and when grown in culture does not become organized into roots, stems, or leaves, but appears to be capable of indefinite growth in its unorganized form.

Turning now to the consideration of some animal virus diseases, one of the most important is *foot-and-mouth* disease of cattle; the disease starts with a rise in temperature during the first two days up to 40° or 41°C, and accompanying the initial fever is a rapid pulse and diminished appetite. Vesicles then start to appear and the fever descends, and usually after 24–36 hours the temperature is normal. The lesions in the mouth are commoner in cattle than in sheep, goats, and hogs, but the buccal mucous membrane may become sensitive and the animal ceases to eat. Lesions develop on the tongue, lip, gums, hard palate, and feet, and sometimes on the udder and teats. Saliva accumulates in great quantities and falls from the mouth when opened. The lesions on the feet may persist for some time, sometimes several weeks. Pigs affected in this way are occasionally seen trying to walk on their knees, and the lameness may be so acute as to prevent the animal from walking. Occasionally cavities develop in the horn of the hoof and these may contain viable virus. As a rule recovery takes place in two or three weeks, although a severe form of the disease is also recognized. In this form, sudden deaths may occur when the lesions are beginning to heal.

There are several strains of the foot-and-mouth disease virus known and there appears to be no cross-immunity between them. The virus is extremely infectious and the disease is dealt with in Great Britain by the slaughter of affected animals and their contacts and incineration of the carcasses.

Dog distemper is a disease which occurs sporadically in every country where there are dogs, and at times very severe outbreaks may pass over a country taking a serious toll of the canine population.

Laidlaw (1930) describes the disease as follows: the onset of illness in the experimentally induced disease is sharply defined by fever and watery discharge from the eyes and nose. The discharge is frequently trivial, but may be profuse. Within 24 hours it may become faintly purulent and the conjunctivae acutely congested. Crusts, composed of half-dried cells, collect at the angles of the eyelids. The temperature rises abruptly at the commencement of the illness and may reach 105°F or even more within 24 hours. After one or two days it subsides to near the normal level of 101·4°F, but it soon rises again. The second rise is slower than the first but much more prolonged. Its duration is

exceedingly variable, and may be as short as 48 hours in milder cases or more than three weeks in more severe. At the onset of the illness the dog usually vomits and refuses all food. With the fall of temperature, appetite returns for a time, to diminish once more as the secondary fever develops. In practically every case there is diarrhoea, and in the secondary febrile period this may be profuse. The poor appetite coupled with the diarrhoea interferes with nutrition and the animal wastes rapidly.

Symptoms from the respiratory system are usually slight. Some cough is common in the period of the secondary fever, but definite bronchitis or broncho-pneumonia does not occur. Definite involvement of the nervous system may take place early or late in the disease, and is of very variable occurrence. The earliest symptom is a sudden attack of semi-consciousness with vigorous chewing movements, accompanied by the secretion of ropy saliva which exudes from the angles of the jaws. Laidlaw points out that dog distemper as seen in veterinary practice differs somewhat from the experimental disease above described, particularly in the incubation period which may be as long as three weeks compared with four days in the experimentally induced disease.

Fowls are susceptible to several virus diseases and two are selected for discussion here, *fowl-pox* and *fowl-pest.*

The disease of *fowl-pox* seems to occur in several forms, although the exact relationship between them does not appear to be known. The two commonest forms are, firstly, the condition known as fowl-pox and, secondly, avian diphtheria. In fowl-pox the skin alone is involved and the head is first infected. A fine, bran-like, grey deposit develops on the comb, ears, and wattles, and later, on parts of the body where the feathers are absent. These initial lesions soon develop into small nodules; these are at first reddish grey but later become greyish yellow. Later still they become brown, dry and firm, their surface is warty and they contain horny or fatty degenerated epithelial cells. They may coalesce and bleed and thick scabs may form over their surface. The margins of the nasal orifices and the eyelids become thickened, and the eyes remain shut. Nodules may also develop on the mucous membranes of the mouth and throat or on other parts of the body.

In the diphtheritic form there is usually no marked general disturbance. The condition frequently begins in the mouth with the formation of a mucous membrane. The membranes are usually

adherent and leave a bleeding surface when torn away; the surface beneath is red and finely granulated. The inflammatory process may extend to the larynx and on to the trachea and bronchi. Respiration and swallowing become very difficult and inspiration is characterized by a whistling noise. Finally the appetite is lost, the animal can neither eat nor drink and must be fed by hand (McKinley, 1929).

Fowl-pest, or Newcastle disease, as it is sometimes called in England, is a severe and extremely infectious disorder and is essentially an infection of the alimentary tract. The onset of the disease is sudden, and affected fowls show a disinclination to move about, preferring to sit or stand quietly in a secluded spot. Early in the course of the illness there is diarrhoea. Gasping for air associated with jerking movements of the head downward and backward is a characteristic symptom. This is caused by large quantities of tenacious mucus which obstruct the posterior nares and pharynx. Many fowls die of suffocation early in the course of the disease. In the latter stages of infection the legs and wings may be completely paralysed and the birds are unable to move. Death usually occurs within a week but may be more rapid; it is not uncommon for every bird in an infected flock to die. Of the fowls that recover from the acute symptoms, a high percentage develop paralysis and only a very few fully recover from this paralysis.

The next four diseases to be described are characterized by growth stimulation and tumour formation. The best known and most studied is a sarcoma of fowls known as the *Rous sarcoma*, so called after its discoverer who described it first in 1910. He showed that the tumour was a spindle cell sarcoma and it was both infiltrative and destructive. In 1911 Rous further demonstrated that the tumour was extremely malignant and possessed a marked tendency to widespread metastasis. Not only was the tumour transmissible to barred Plymouth Rock fowls by inoculation with tumour material, but also with bacteriologically sterile Berkefeld filtrates of tumour emulsion. Rous also discovered that the tumour could be transmitted to susceptible fowls with dead cells killed by desiccation or by 50 per cent glycerol.

Fowl leukaemia is of interest because of the resemblance which it bears to the leukaemia of man and because it is one of the few examples of a blood disease which can be transmitted experimentally. Ellermann (1921), quoted by Bedson (1930), recognizes three clinical types of fowl leukaemia: the lymphatic, the myeloid, and the anaemic. In the *lymphatic* type there are few clinical symptoms; the blood picture

is normal but the liver may be enlarged up to eight times its normal size and mottled in appearance. The spleen is also enlarged. In the *myeloid* type, the birds do show some recognizable symptoms during life. They appear ill and off their feed, and their combs are pale. The blood picture is definitely pathological with an enormous increase in the number of leucocytes. Counts of 200,000 to 600,000 per cu.mm. are met with, the normal count in the hen being about 30,000 per cu.mm.

In the *anaemic* type the outstanding feature is the yellow colour of the comb and other visible epidermal structures. This yellow colour is not due to bile-pigment but to the normal lipochrome of the hen, and is therefore an exaggeration of a normal process. The underlying pathological condition is an increase in the fatty substances in the plasma, the vehicle of the lipochrome (Bedson and Knight, 1924). The total blood count is low, one million or less, as against the normal figure of three millions. The liver is enlarged and paler and yellower in colour than normal. The spleen is enlarged up to four or five times its normal size.

Fowl leukaemia appears to be a very fatal disease and the fowl seems to be the only susceptible animal.

Of these tumour-forming viruses in fowls, Rous (1943) writes as follows—

> Viruses are responsible for a wide variety of naturally occurring mesodermal growths in the domestic fowl—fibromas, sarcomas, myxomas, osteomas, chondromas, lymphoid tumours, and so forth—as well as for leukaemias and an endothelioma. Carcinomas occur frequently in chickens but are usually overlooked, and there has been little effort to determine their cause, and this little ineffectual. . . . The chicken tumours that yield virus are typical neoplasms in all respects, even in metabolic peculiarities, and like them they appear hither and yon in the fowl community, giving no indication of any connexion with one another. The causative viruses are as various as the tumours, each engendering growths of the sort from which it was originally procured, and growths of such sort only.

Rabbits are also susceptible to virus diseases which take the form of papillomas on the skin and an *infectious papillomatosis* of rabbits has been described by Shope (1933). These papillomas or warts are frequent in Kansas, U.S.A., where about one animal in every twelve trapped may be found to carry them. The warts vary in size and may be quite small, or large onion-shaped or jagged growths. The number of warts on individual animals varies, though exceptional cases have occurred where the body was entirely covered with warts, enough,

as Shope says, to fill a 200 c.c. flask. The most common sites for the warts appear to be on the inner aspect of the thighs, the abdomen and on the neck and shoulders. The warts are black or greyish black in colour, well keratinized, and the upper surfaces are frequently irregular or fissured. The lateral surfaces of the warts appear vertically striated because each individual wart is composed of closely-packed and almost homogeneous vertical strands of tissue. Sections of warts show a white or pinkish white fleshy centre, and the upper portions of its lateral surfaces are greyish black and keratinized. Shope found that the disease was easily transmissible to healthy rabbits by dropping some of a suspension of warts upon a portion of the skin which had been shaved and lightly scarified by means of a needle or a piece of sterilized sand-paper.

Infected rabbits exhibited no clinical symptoms and appeared to be in normal health. The virus was found to pass Berkefeld filters of V, N, and W porosity.

Rous (1943) describes how this rabbit papilloma virus is capable of infecting the epidermal tumours which can be produced experimentally by means of tar and other chemical carcinogens. It is significant that some of the tar papillomas undergo an abrupt change into carcinomas when the virus lodges in them; for the alterations which take place then resemble in all obvious respects those occurring when virus-induced papillomas become cancerous.

Before concluding this discussion of tumour-forming viruses, it may be of interest to mention an unusual host for this type of virus, *Rana pipiens*, the leopard frog. Lucké (1938) describes how this frog is commonly affected with a carcinoma of the kidney. It is a particularly interesting tumour because the cell nuclei frequently contain large acidophilic inclusions which are similar to those found in herpes and certain other diseases known to be due to viruses. The tumours always originate from the renal epithelium and exhibit its morphology; they range in aspect from benign adenomas to malignant carcinomas and behave accordingly. The causative agent is strictly specific, acting only on leopard frogs and the renal epithelium.

There is a group of interesting viruses which attack only the larval forms of Lepidoptera and some Hymenoptera (sawflies) and give rise to what are known as *Polyhedral diseases*. The outstanding characteristic of these diseases is the formation within the body of the host of enormous numbers of many-sided crystal-like bodies from which the name of this type of disease is derived.

These viruses attack many different species of larvae but are extremely host-specific and instances of a virus from one species attacking the larvae of another are rare.

Among the insects infected with this type of virus are the following: *Bombyx mori*, the silkworm, *Lymantria dispar*, the gipsy moth, *Lymantria monacha*, the nun moth, *Arctia caja*, the tiger moth, *Abraxas grossulariata*, the currant moth, *Ptychopoda seriata*, *Neodiprion sertifer*, the pine sawfly, and *T. bisselliella*, the clothes moth. A description of the disease as it develops in *A. grossulariata* will suffice, since it is fairly typical. External signs of the disease appear in an experimentally induced infection after about ten days, and usually take the form of a dark discoloration lying dorsally near the head; this is followed soon after by a blackening of the whole body and death of the larva. Sometimes the skin ruptures before death and liberates vast quantities of polyhedral bodies (Plate VI). It is an interesting fact that an infected larva continues to feed and live an apparently normal life right up till the last stage of the disease when the skin becomes discoloured. Sections of infected larvae show that advanced disorganization of the internal tissues has taken place some time previous to the first external sign of the disease. The polyhedral bodies are first observed in the nuclei of blood cells, hypodermal and fat cells which become greatly enlarged and are packed with large numbers of the polyhedra (Plate VII). The nuclei swell up, becoming three or four times the size of normal nuclei and completely filling the cell. If these polyhedra are placed in a weakly alkaline solution and observed under the $\frac{1}{12}$ oil immersion with dark ground illumination, it is possible to see the actual virus particles moving rapidly about inside the disintegrating polyhedral body. Occasionally, impelled by Brownian movement, they pass right outside.

Shortly after death all the tissues disintegrate and it is very difficult to lift up a caterpillar which has died from polyhedral disease without the skin rupturing and the contents being dispersed. Indeed, each time the caterpillar is touched the skin bursts again, so that in the end the insect must be scraped up rather than lifted bodily.

In the silkworm, infected caterpillars in the later stages of the disease become sluggish and cease to eat, whilst lemon-yellow patches develop in the skin. It is from this yellow colour that the name *silkworm jaundice* has been derived. Before death the skin assumes an opaque, shiny, and yellowish appearance.

Not very much intensive study has as yet been carried out on the

virus diseases of insects, and compared with the virus diseases of other types of organisms this branch of virus work has been much neglected. There is a good deal of evidence for the existence of other virus diseases attacking the larval forms of insects in which the characteristic polyhedral bodies are not formed, their place being taken by large numbers of very small granules. The exact significance of these granules is not known.

Before concluding this brief survey of representative virus diseases, mention must be made of the bacterial viruses or "bacteriophages" as they used to be called.

The destruction or "lysis" of bacteria by some agent which appeared to be of a virus nature was first observed in 1915 by Twort. He made the chance discovery that colonies of micrococci growing on agar as a contaminant from glycerolated vaccine virus tended to become "glassy" or transparent. Sub-cultures from such colonies grew for a time and then became "glassy" in their turn (upper photograph, Plate VIII).

Twort considered that some virus-like agent must be concerned in the destruction of the bacteria since filtrates of "glassy" material diluted 1 : 10,000,000 were still active. Two years later d'Herelle described a similar phenomenon in growing cultures of *B. dysenteriae*. Since that time the bacterial viruses have been most intensively studied, especially in the U.S.A., and a vast literature on the subject has grown up. Since this chapter is concerned only with the description of virus diseases and not of the viruses it will be relevant to describe some effects of the viruses on the bacteria.

d'Herelle had observed that soon after the exposure of bacteria to virus, the former begin to swell and finally burst, due to internal pressure which he thought was exerted by the parasites multiplying within the cells. A study of the intimate mechanism of the lysis of bacteria by the virus was made by Bronfenbrenner, Muckenfuss and Hetler (1927), who took a cinematographic record of lysis of *B. coli* on agar at 37°C. Bronfenbrenner describes the process as follows—

> The progress of lysis was photographed by means of an automatic camera at a rate of 25 exposures per minute. The record shows that, after a short period of lag, bacteria began to multiply at a rate notably exceeding that of normal bacteria photographed under similar conditions but in the absence of phage (virus). This stimulating effect of phage has been reported by a number of investigators. Due to the rapidity of growth, many cells failed to divide completely and gave rise to filaments having a length of from 10 to 20 times that of an average normal cell. By the end of the first hour

of growth, occasional cells began to show signs of swelling, and by the end of the third hour the great majority of cells in the field appeared more or less swollen, a few among them reaching several times their normal dimensions. Filaments swelled as well as ordinary bacteria. The swelling continued slowly until about the fifth hour, when, one by one, bacteria began to disappear suddenly and quickly.

Before leaving the bacterial virus diseases it will be of interest to refer to the most recent work by Wyckoff (1948) who examined the lysis of bacteria by viruses with the aid of the electron microscope. He found that the phenomena accompanying lysis depended in striking fashion on the strain of bacteriophage. Sometimes the fragments of lysed bacteria are of a granular texture, but much of the protoplasm freed by lysis seems to be filamentous. Photographs taken, at a magnification of about 40,000, of a colon bacillus infected with T_3 strain of bacteriophage show a regular pattern of concavities extending over the entire surface of the organism.

CHAPTER V

ISOLATION AND PURIFICATION OF SOME REPRESENTATIVE VIRUSES

For some years previous to 1935 several attempts to isolate the virus of tobacco mosaic from the plant sap had failed and the first to succeed in this task was Stanley (1935) who used salt-precipitation methods. Since that time much progress has been made in the isolation of viruses, not only of plants but of animals as well, and some of the methods used are briefly described in this chapter. It is proposed to discuss the isolation and crystallization of two representative plant viruses and the purification of three animal viruses, including one insect virus, and one bacterial virus.

Plant viruses which are suitable for purification must be fairly stable, that is they must resist denaturation by low temperatures and by oxidation when expressed from the plant with the sap. They must also be present in high concentration in the host plant; this is very important because it is of little use attempting to isolate a weakly infectious and unstable virus.

The two main methods employed for the purification of plant viruses are the chemical-precipitation method and isolation by high-speed centrifugation. The latter is not so drastic as chemical precipitation and can be used with viruses which are less stable and concentrated. Sometimes it is preferable to use a combination of the two methods.

The following is a brief outline of the technique for isolating the virus of *tobacco mosaic* by chemical methods. The leaves are harvested from young, systemically infected, tobacco plants; young plants are preferable to old since the latter contain much tarry material which it is difficult to eliminate. The leaves are frozen overnight, minced in an ordinary meat mincer and then thawed. The freezing and subsequent thawing seem to make the sap easier to handle; the leaf pulp and sap are then pressed by hand through muslin, in order to remove large particles. More sap may be extracted from the "cake" by means of a press, either a hand or hydraulic press according to the quantities used. It has recently been shown (Bawden and Pirie, 1946) that more virus can be obtained from the dried pulp by digesting it

with the enzymes from snail gut. The next step is to clarify the crude extracted sap; this can be done by heating to 55°C for a few minutes or, alternatively, by adding 2–4 per cent disodium phosphate. The sap is then centrifuged for about 15 minutes at 3000 r.p.m. to remove the bulky coagulum which is discarded. To the clear supernatant fluid is now added half its volume of a saturated solution of ammonium sulphate to give one-third saturation. The virus is thrown out of solution and comes down as a white flocculent precipitate. This precipitate is spun down on the centrifuge and re-suspended in a volume of water equal to about one-fifth to one-tenth of the original volume of sap; this is centrifuged again to remove any insoluble matter. The virus is then re-precipitated either by acidifying the solution with a 10 per cent solution of acetic acid or by one-third saturation, again with ammonium sulphate. The precipitated virus is centrifuged off and re-suspended in a small volume of water and dialysed against running water. When free from ammonium sulphate the solution should be centrifuged again to get rid of insoluble matter. The purified virus solution should be opalescent and almost colourless. If sufficiently concentrated such a solution will, on standing, separate into two layers; the lower layer is the more concentrated and is spontaneously birefringent. The upper layer is not birefringent when stationary but if gently shaken it exhibits anisotropy of flow.

If acid or ammonium sulphate is added to the solution, the virus comes out of solution as a precipitate with a characteristic satin-like sheen. Under the microscope this precipitate is seen to be composed of needle-shaped paracrystals. These needles were first isolated by Stanley in 1935; they are not, however, true three-dimensional crystals.

The virus of *turnip yellow mosaic* is more easily purified than that of tobacco mosaic or probably any other plant virus and forms true three-dimensional crystals. The leaves are harvested from infected plants of either turnips or Chinese cabbage and frozen overnight. Curiously enough much more virus is obtainable from old, rather hard, turnip plants growing in the field, than from young, sappy, Chinese cabbage grown in the glasshouse. This is the reverse of what happens with tobacco plants infected with the tobacco mosaic virus. The leaves are minced and thawed as before and the sap is expressed through muslin; to each litre of sap is added exactly 300 ml of 90 per cent alcohol. This clarifies the sap and the precipitate is removed by centrifuging, the virus remaining in the supernatant fluid. It is

important that the exact amount of 90 per cent alcohol should be added, as otherwise the virus is liable to be thrown down and will be lost with the precipitate. To the supernatant liquid is now added half a volume of saturated ammonium sulphate solution to bring it to one-third saturation.

This precipitates the virus in the form of small octahedral crystals (lower photograph, Plate VIII). Further purification may be effected by re-crystallization from salt solution. A variety of salts are suitable for this purpose, but all deposit the virus in the form of octahedra.

An alternative method is to re-crystallize the virus from .20 per cent ethyl alcohol at a *p*H of 3·8. This must be carried out in the cold at a temperature of 5°C or less. Under these circumstances the virus does not form octahedra but is deposited as fine needles which frequently form globular clusters (Markham and Smith, 1949).

In the purification of animal viruses the worker is handicapped because from the nature of his material he is not always able to obtain large quantities of virus in the manner available to plant-virus workers. A good deal of work on the purification of influenza virus was carried out during the war chiefly with the aim of obtaining vaccines and some of these methods as carried out by Stanley (1944) are briefly described.

The PR 8 strain of influenza virus was used in the work and the starting material was in the form of frozen and dried allantoic fluid from the hen's egg in which it had been cultured. The virus was isolated originally from a ferret, passed many times in mice and tissue culture and finally several times in chick embryos. The material was concentrated and purified by four general methods involving (*a*) differential centrifugation, (*b*) adsorption on, and elution from, adult chicken red cells, (*c*) elution of the precipitate formed on freezing and thawing of allantoic fluid, (*d*) adsorption on and elution from embryonic chick red cells.

In method (*a*) 320 c.c. of infectious allantoic fluid was centrifuged in the ultracentrifuge at 24,000 r.p.m. for two hours. At the end of that time the pellets were found to contain 97 per cent of the virus activity. The pellets of sedimented protein were then suspended in 35 c.c. of 0·1 M phosphate buffer at *p*H 7 and centrifuged at a low speed of 3000 r.p.m. to remove the insoluble protein. The 35 c.c. of supernatant fluid were again centrifuged for two hours at 24,000 r.p.m. The upper two-thirds of the supernatant fluid were removed

and found to contain no activity. The sedimented protein was suspended in 5·4 c.c. of buffer and on centrifuging at slow speed about 0·6 mg of insoluble protein without activity was removed.

In method (*b*) to 320 c.c. of allantoic fluid similar to that used in method (*a*) and cooled to 4°C were added 14 c.c. of a 23 per cent suspension of adult chicken red cells. After standing for five hours at 4°C, the preparation was centrifuged for 10 minutes at low speed to separate the red cells. The supernatant was removed and found to have about 30 per cent of the activity, so that about 70 per cent of the original activity was adsorbed on the red cells. Next the red cells were eluted with 32 c.c. of 0·1 M phosphate buffer at *p*H 7 for 2½ hours at 37°C and then centrifuged at low speed; the red cells were then eluted a second time.

In method (*c*) the remaining 320 c.c. portion of allantoic fluid was transferred to celluloid centrifuge tubes of 90 c.c. capacity, frozen overnight in a CO_2 ice-box, and then thawed at room temperature for about 2½ hours. Care was taken that the temperature of the liquid did not rise above about 3°C. The fluid was centrifuged at 3000 r.p.m. in a cold room and the precipitate was collected and extracted with 32 c.c. of 0·1 M phosphate buffer at *p*H 7 for 30 minutes at about 23°C. After centrifugation at low speed, the residual precipitate was again extracted as described above to yield a second extract. The first extract contained most of the virus.

In method (*d*) eighty-nine infected chick embryos were taken directly from the incubator after 36 hours' incubation, opened and the blood vessels torn. The bloody allantoic fluid was harvested within a few minutes and collected in iced centrifuge tubes. The 570 c.c. of fluid obtained by this procedure were allowed to stand at 4°C for 5 hours and then centrifuged at low speed in the cold room. The embryonic red cells were eluted twice for 2½ hours at 37°C with two 57 c.c. portions of buffer.

The supernatant liquid from the original allantoic fluid, after adsorption with embryonic cells, was found to possess some activity. In order to recover this activity, the preparation was treated with 25 c.c. of a 23 per cent suspension of adult chicken red cells for 5 hours at 4°C. The red cells were then removed by centrifugation at low speed and eluted with 57 c.c. of buffer for 2½ hours at 37°C.

Of these four methods of purifying the influenza virus, method (*a*) gave a final preparation with three times the activity of method (*c*) which gave the next highest infectivity.

A method of obtaining highly purified preparations of an animal virus by means of centrifugation alone has recently been described by Taylor (1946) and the following description is taken from his account of his work. The virus in question is that of the *Shope rabbit papilloma* which produces large warty outgrowths on the skin of infected cottontail rabbits. The sources of virus were the naturally occurring papillomas obtained from rabbits trapped in Kansas, U.S.A.

The warts were removed from the animals in the field, covered with a mixture of glycerol and 0·9 NaCl in 8-oz bottles and stored at a temperature of 2–8°C. The growths were collected over a long period and stored until sufficient material was obtained for the work. In a typical experiment, 150 g of warts were freed of hair and extraneous tissue and broken up in about 100 ml of 0·9 per cent NaCl solution. The pulp was made up to a volume of 2 l (a 7·5 per cent tissue suspension) and set aside at 2–8°C to extract overnight. The suspension was then mixed with 50 g of No. 512 celite and filtered through a thin mat (2–3 mm) of No. 503 celite. The resulting, slightly turbid, filtrate was passed into the Sharples supercentrifuge through a No. 24 gauge hypodermic needle at a constant rate of 500 ml per hour. A centrifugal field of 62,000*g* was maintained at the inside bowl periphery (50,000 r.p.m.). The extract was followed with 500 ml of a solution containing 0·13 M NaCl and 0·05 M phosphate buffer, *p*H 6·5, to displace the final 50 ml of extract and to wash the sedimented virus. As the speed of the bowl slowed, the lower inlet boss was stoppered.

The bowl, containing the concentrated virus in a volume of 50 ml, was removed from the machine and put in the cold room overnight. The next morning, after thorough shaking of the bowl, the 50 ml of virus suspension were washed into a 100 ml centrifuge tube with 20 ml of the buffer saline solution. This was spun in an angle centrifuge for 15 minutes at 3000*g* and the supernatant fluid decanted. The soft, insoluble sediment was washed twice with 25-ml portions of buffer saline solution, and the wash fluids were added to the initial concentrate. The virus recovered from an initial volume of 2 l of crude extract was now in a volume of 120 ml. This was next subjected to a single cycle of centrifugation in the vacuum-type air-driven ultracentrifuge (5000*g* for 5 minutes, 50,000*g* for 1 hour), after which the pellets were taken up in 6·5 ml of the buffer saline solution. Large aggregates were eliminated by low speed centrifugation in the vacuum ultracentrifuge at 5000*g* for 5 minutes.

In one such experiment 50·4 mg of virus were obtained, a yield of 0·33 mg of virus from 1 g of warts.

Satisfactory yields of purified rabbit papilloma virus can thus be obtained by preliminary sedimentation with the Sharples super-centrifuge from large volumes of extracts of virus warts, followed by repeated high and low speed spinning in the vacuum-type air-driven ultracentrifuge.

The next example of virus purification is given by one of the virus diseases of insects, known as *polyhedral* diseases, and here the technique of isolation is rather different. In this type of disease, most of the virus is contained inside the polyhedral bodies and the method of isolation is based mainly on the work of Bergold (1946) and refers to a polyhedral disease of the caterpillars of the gipsy moth (*Lymantria dispar*). The dead and semi-liquefied bodies of the larvae are collected and put together into a glass vessel, preferably tall and narrow, with water or saline, and allowed to stand for some weeks. Part of the object of this is to allow bacterial decomposition of the tissues. During this time, the polyhedra sediment to the bottom, after which the fluid and decomposing bodies can be decanted off. The polyhedra should then be washed thoroughly with water and drawn through a piece of fine muslin with a suction pump. If properly clean, the deposit of polyhedra should be white. The next step is to dissolve the polyhedra to free the virus and this must be done at an alkaline *p*H of 9 or 10. About 35 mg of dry polyhedra are put in a clean dry flask and 7 c.c. of alkali are added, using 0·008 M Na_2CO_3 and 0·05 NaCl. This is left to stand, with the vessel closed, for two or three hours. The solution should become fairly, but not too, clear. The next step is to spin out the undissolved material at 6000 r.p.m. for 5 minutes and decant the supernatant fluid which contains the polyhedra protein plus virus. The fluid must then be spun in a centrifuge at 10,000 r.p.m. for one hour, which gives a small bluish-white precipitate. The supernatant should be decanted very carefully and 7 c.c. of glass-distilled water added to the precipitate; it can then be left for one hour but the precipitate should dissolve in 5–10 minutes giving an opalescent fluid with little or no insoluble matter. This should be spun again at 10,000 r.p.m. for one hour (7000*g*), after which the supernatant should be quite clear, with all the virus thrown down. The virus is then dissolved in water once more and the final solution should be opalescent. It is important to keep such a virus solution at *p*H 7 or above and it must not be

frozen, otherwise the virus is thrown out of solution and will not re-dissolve.

Finally, a short description is given of the purification of a bacterial virus, the T_2 or P.C. bacteriophage of *Escherichia coli*. The method is that of Hook, Beard *et al.* (1946) and quotation from their work is freely made. The purification was carried out on phage-cultured bacteria grown both in broth and synthetic media.

In the production of the phage for concentration and purification, batches of 7·5 to 15 l, distributed in 1500 ml volumes in 2-l Florence flasks proved convenient. Seed inoculation of bacteria for the large volumes was prepared in 50 ml of the respective media in 125-ml Erlenmeyer flasks inoculated with 0·1 ml of an 18–24 hour broth culture of *Escherischia coli* and incubated at 37°C for 18 hours. Stock phage containing 10^{10} lytic units per ml was added in the ratio of one phage particle to 400–700 bacteria, and the flask was shaken and allowed to stand for 5–7 minutes. To each 1500-ml volume of medium previously warmed to 37°C, there was added 30 ml of the phage-bacteria suspension.

The flasks were incubated for 8 hours at 37°C with vigorous mixing by hand every 15 minutes. The individual batches of nutrient broth cultures were pooled immediately and stored in the ice-box for 7 to 14 days before processing. The nutrient broth cultures usually were completely lysed at the end of 8 hours but the synthetic medium cultures took longer.

Whilst the phage can be readily concentrated by the centrifugation of freshly-lysed nutrient broth cultures of 8 hours' incubation, purification at this stage is very difficult. The fresh lysates contain much mucoid material and the phage particles are apparently trapped in, or coated with, the slimy substance. As a result, efforts to remove this substance by filtration through candles or slow-speed centrifugation are liable to result in the loss of 60–80 per cent of the phage activity.

This difficulty can be got over by allowing the lysates to stand in the refrigerator for 7–14 days. During this period a granular or flocculent precipitate will sediment, taking down with it much of the slimy substance. Elimination of bacteria and bacterial debris, as well as the flocculated mucoid material, can now be accomplished by low-speed centrifugation in the Sharples centrifuge. Later work, however, has shown that filtration of the broth lysates through 10-in. 6- or 7-lb Mandler candles removes the extraneous material with less loss of virus than does the centrifugal clarification.

For the initial concentration of the virus, the clarified lysate is passed through the 50-ml concentration bowl (20) of the Sharples centrifuge at a rate of 2 l per hour. The bowl speed should be 45,000 r.p.m. (49,000*g*).

After washing with saline and cooling overnight the suspension from the Sharples should be opalescent; it shows a slight yellow colour due to a small amount of mucoid material still remaining. This can be removed by spinning on an angle centrifuge at 2000*g* for 10 minutes. The supernatant from this should show a bright blue, opalescent, Tyndall effect, and should be devoid of yellow colour. The next step is to put it in the chilled rotor of the ultracentrifuge and spin at 20,000*g* for 40 minutes. The ultracentrifuge concentrate, when re-suspended, should be strongly opalescent and blue with sharp high-lights when viewed by transmitted light.

CHAPTER VI

PHYSICAL AND CHEMICAL PROPERTIES OF VIRUSES

In the previous chapter we examined briefly some of the methods of purifying and isolating viruses, and in this chapter we shall consider some of the chemical and physical properties of such purified viruses.

Tobacco Mosaic Virus

The virus of tobacco mosaic is one of the most infectious and stable viruses known, and has been found to be still infectious after being kept in extracted sap for a period of thirty-four years. It occurs in high concentration in the tobacco plant and about 2 g per litre of sap can be obtained; additional virus is liberated by incubating the leaf residues with snail-gut enzymes (Bawden and Pirie, 1946). The amount of virus, however, which occurs in plants affected with some mutant strains of tobacco mosaic virus is considerably less than that referred to above.

If a purified solution of tobacco mosaic virus of about 1–2 per cent is left standing, it separates into two layers of which the lower is the more concentrated. Under polarized light, the lower layer is spontaneously birefringent whilst the upper layer only becomes so when shaken. This separation into layers occurs when the concentration is too great to allow free movement of the particles which arrange themselves into small boat-shaped drops called "microtactoids." These are of slightly higher density and so fall to the bottom. Bernal and Fankuchen (1937) found this bottom layer to be a jelly with all the rods the same distance apart, and Bernal considers each rod of the tobacco mosaic virus to be itself a small crystal.

If the bottom layer is allowed to dry gradually in a thin cell, the distance between the rods gets less and eventually the rods dry, forming a dry gel. The centres of the particles are separated by 15·2 mμ, but on drying completely the distance is reduced to 15 mμ and this is taken as the diameter of the rod.

On treatment with dilute alkalis, the tobacco mosaic virus particles tend to split down into smaller and smaller particles, and sub-units tend to form with some regularity (Schramm, 1947), but these small

sub-units are not, of course, infectious, and the nucleic acid is split off most of them.

When completely dry, the gel is horny, yellowish in colour, and only feebly birefringent. Wyckoff has shown that under the electron microscope the wet gel does not appear to be the hexagonally ordered array, postulated by Bernal and Fankuchen, but consists instead of a flat sheet, or several layers of sheets (Plate XV).

Although the tobacco mosaic virus, when purified, does not form three-dimensional crystals, infected plants do contain true crystals in the form of hexagonal plates, first described by Iwanowsky and called by him "striate material." When these plates are touched in the cell with a micro-manipulator, they break up into the fine needles first described by Stanley, and it is thought that they are a complex of virus and some other component. According to Kausche crystals of this type can be produced by the action of plant sap upon the ammonium sulphate precipitate of the virus, but no one has confirmed this rather unlikely statement.

The tobacco mosaic virus is stable over a range of *p*H 1·5–8·5 but is not stable to alkali; it precipitates at its isoelectric point, *p*H 3–4.

The virus consists entirely of protein and nucleic acid, the latter being about 5–6 per cent of the total.

It is not possible to separate the nucleic acid without denaturing the protein and thus destroying the activity of the virus. Any treatment which denatures the protein separates off the nucleic acid, and there is a great deal of evidence which suggests that the nucleic acid is essential for multiplication of the virus.

The nucleic acid is of the ribose type and in 1947 Schwerdt and Loring isolated the brucine salts of uridylic acid and guanylic acid from hydrolysed nucleic acid. The elementary composition of nucleic acid is as follows: C 35 per cent, H 42 per cent, N 15 per cent, P 9 per cent, Purine N 8·5 per cent, CH_2O 31 per cent. There seems, however, to be too little carbohydrate.

The protein component of tobacco mosaic virus is unusual; instead of being the basic protein of the histone or protamine type which one might expect in a nucleo-protein, the protein component is acidic, with an isoelectric point about *p*H 5·3. The general composition resembles that of casein (Chandler *et al.*, 1947), and has no preponderance of basic amino acids.

There are sixteen amino acids in all (Knight, 1947), the major one being arginine 9·8 per cent, the others are lysine 1·47 per cent, aspartic

acid 13·5 per cent, glutamic acid 11·3 per cent, which accounts for the acidity of the protein, cysteine (no cystine) 0·69 per cent, glycine 1·9 per cent, isoleucine 6·6 per cent, leucine 9·3 per cent, phenylalanine 8·4 per cent, proline 5·8 per cent, serine 7·2 per cent, threonine 9·9 per cent, tryptophane 2·1 per cent, tyrosine 3·8 per cent, valine 9·2 per cent.

There is no histidine and no methionine; on the other hand, a distantly related strain of tobacco mosaic virus, obtained from plantain, contains 0·72 per cent histidine and 2·2 per cent methionine, both of which are absent in the type virus.

Two other distantly related viruses, known as cucumber viruses 3 and 4, contain very little glutamic acid and no sulphur-containing amino acids. It is also possible to differentiate between the type virus, the plantain virus and cucumber viruses 3 and 4 on their aromatic amino acids, irrespective of the host plant from which they are taken. (Table II.)

TABLE II

AROMATIC AMINO ACIDS AND PHOSPHORUS IN STRAINS OF TOBACCO MOSAIC VIRUS AND IN CUCUMBER VIRUSES 3 AND 4

Virus	Tyrosine	Tryptophane	Phenylalanine	Phosphorus
	per cent	per cent	per cent	per cent
Tobacco mosaic .	3·8	4·5	6·0	0·56
Yellow aucuba .	3·9	4·2	6·3	0·52
Green aucuba .	3·9	4·2	6·1	0·54
Plantain strain .	6·4	3·5	4·3	0·53
Masked strain .	3·9	4·3	6·1	0·54
J 14 D 1 . .	3·8	4·4	6·1	0·55
Cucumber virus 4 .	3·8	1·4	10·2	0·54
Cucumber virus 3 .	4·0	1·5	10·0	0·56

(*After Knight and Stanley*)

Tomato Bushy Stunt Virus

This virus, first described by Smith (1935), was also the first virus to be isolated in a three-dimensional crystal form (Bawden and Pirie,

1938). Although it now seems to have disappeared completely from the tomato crops of this country, the virus is to be found in laboratories in many parts of the world where, because of its scientific interest, it has been intensively studied.

Bawden and Pirie purified this virus in the first instance by heating the sap extracted from young infected tomato plants to 60°C, spinning off and discarding the precipitate and adding ammonium sulphate. However, since heating greatly reduces the infectivity of the virus without apparently causing any other change in its nature, the method of purification was revised and the heating omitted. Stanley (1940[a]) used *Datura* plants as a source of virus in preference to tomatoes.

Purified solutions of tomato bushy stunt virus are opalescent and show no flow birefringence, such as is characteristic of tobacco mosaic virus, since the particles are spherical. The virus is soluble in water and dilute salt solutions over the whole *p*H range in which it is stable and it does not, therefore, precipitate at its isoelectric point which is *p*H 4·1. On the addition, at room temperature, of saturated ammonium sulphate to the opalescent virus solution, an amorphous deposit separates out. This is better carried out at 0°C than at room temperature since the virus is more soluble in the cold.

The crystals produced by this method are rhombic dodecahedra, but Cohen (1942) has shown that by the use of heparin and other substances in the crystallization process the virus forms prisms which appear to be isotropic.

The elementary analysis of purified preparations made by ammonium sulphate precipitation has given the following values: carbon 48·5 per cent, hydrogen 7·7 per cent, nitrogen 16·1 per cent, sulphur 0·6 per cent, phosphorus 1·5 per cent. Nothing has yet been published on the amino-acid composition of the protein part of the virus.

Nucleic acid of the ribose type has been isolated from a preparation of bushy stunt virus purified by centrifugation and the carbohydrate and phosphorus are largely present in this form. There appears to be about 17 per cent of nucleic acid.

No enzymes are known which will digest the bushy stunt virus and it can therefore be incubated with trypsin to assist the purification processes.

The infectivity of the purified tomato bushy stunt virus is 10^{-8} g per millilitre.

Turnip Yellow Mosaic Virus

The methods for isolating and purifying this virus have been described in the preceding chapter and the purified virus crystallizes in the form of small octahedra (Plate VIII, lower photograph). This virus is an unusual one in some respects and chemical studies upon it are therefore of great interest. Virus preparations made by the alcohol and ammonium sulphate precipitation methods described in Chapter V are highly infectious and are quite uniform in composition. They contain about 15 per cent nitrogen, 2·15 per cent phosphorus, and 5 per cent of purine-bound pentrose, the latter two being entirely in the form of nucleic acid. The isoelectric point of the virus in solution is *p*H 3·75. The virus is unstable to weak alcohol and the solution is denatured by $33\frac{1}{3}$ per cent alcohol. The yield of virus from infected plants varies but may be as much as 1 g per litre. The infectivity is 10^{-9} g per millilitre.

The most interesting point about this virus is the existence in the infected plant of two components which differ in their sedimentation constant. This means, therefore, that when a solution containing more than 10 mg per millilitre of virus protein is spun on a Sorvall S.S.1 centrifuge it separates off into two layers, a "top" and a "bottom" component. The boundary between these two layers is sufficiently stable to allow the top component to be removed by a pipette. The top and bottom components are similar, if not identical, in the following respects. Both crystallize from salt solutions in octahedra (Plate IX) and under the electron microscope are spheres of similar size. In solution, too, they seem to be the same size, and both are resistant to digestion by pancreatic enzymes.

The bottom component, however, is the only one containing nucleic acid, and the absorption spectra of the two are consequently very different. Although complete amino acid analyses are not yet available, preliminary paper chromatography using several solvents indicates that both proteins contain the same amino acids in similar proportions. All the evidence suggests that the protein parts of both components are identical and that the bottom component differs only in having some 28 per cent of pentose nucleic acid by weight embedded in it in such a way that it takes no part in forming the electrostatic surface of the molecules. The comparatively acid isoelectric point of both proteins seems to be due to the relative preponderance of the dicarboxylic amino acids in the protein.

The top component is therefore the first native virus protein which has been obtained free of nucleic acid.

The fact that the top component is non-infectious is direct evidence that nucleic acid is essential for the multiplication of plant viruses since the two components seem otherwise identical.

The origin of the top component is at present obscure; it may represent a stage in the synthesis or the breakdown of the nucleoprotein, or might even be the result of an abortive attempt at multiplication (Markham and Smith, 1949).

Plant and animal viruses have a common chemical background in that both appear to contain nucleic acid and protein; plant viruses, however, contain ribonucleic acid whereas those animal viruses examined so far contain either ribonucleic or desoxyribonucleic acid. Some animal viruses appear to be more complex and also contain fats.

Influenza Virus

There are two main strains of influenza virus, known as A and B, and they do not immunize against each other. There is a third strain, swine influenza virus, which is related to A; this is thought to be a survival of the virus which caused the great influenza pandemic of 1918–19, having become adapted to the pig and persisting in it ever since.

The methods of purifying influenza virus have been briefly described in Chapter V; there are two main techniques, one by sedimenting out of solutions in the Sharples centrifuge and the other by adsorbing the virus on to chicken red cells and eluting with phosphate at *p*H 7·1. The yield is fairly small; with virus A it is 40 mg per litre of extra-embryonic fluid and with virus B about 60 mg per litre.

Investigations on highly purified preparations of influenza virus have revealed the presence of an amount of carbohydrate apparently greater than that accountable for in the nucleic acids. Knight (1947) obtained carbohydrate-rich fractions from highly purified samples of PR 8 and Lee influenza viruses and subjected them to analysis. In each case, the carbohydrate appeared to be a polysaccharide composed of mannose, galactose, and glucosamine units.

Electrophoretically, the virus is not very homogeneous; it is insoluble at its isoelectric point of *p*H 4·5–5·5.

The elementary composition is C, 52 per cent; N, 9·7 per cent; P, 0·85 per cent; Ribose, 0·71 per cent; CH_2O, 6·1 per cent or 13 per cent; Fat, 24 per cent. According to Taylor (1944) there is 2 per

cent of thymus nucleic acid in virus A and 3·7 per cent in virus B, and no ribose nucleic acid. But Knight (1947) found about 2·3 per cent of ribose nucleic acid present.

Vaccinia Virus

Compared with those plant viruses which have been studied and some animal viruses, vaccinia virus is very large and approaches more nearly the conception of an organism.

Under the optical microscope, with the dark-ground field, the virus particles appear to be spheres, but electron micrographs (*see* Chapter VII) show the particles to be brick-shaped with a "nucleus" of denser material. They vary in size and may be about half as big as a small coccus.

The chemical composition of vaccinia virus is 0·6 per cent P, 15·3 per cent N, 2·2 per cent neutral fats, 2·2 per cent phospho-lipoid, and 1·4 per cent cholesterol, giving a total of 5·7 per cent lipoid. It is not certain that these last two are necessary constituents of the virus. There is present about 5·6 per cent nucleic acid; this nucleic acid is not acted upon by ribonuclease and gives the reactions for thymus nucleic acid. Adenine and guanine have been identified from hydrolysates of the nucleic acid. There is a small quantity of copper present which does not seem to be an impurity since it cannot be removed by any mild method and is actually concentrated during the purification. The virus also seems to contain the enzymes phosphatase, catalase and lipase though it cannot be said for certain that these are part of the virus as the latter is capable of picking up these enzymes from solutions. Vaccinia virus is not acted on by trypsin.

The *rabbit papilloma* virus of Shope is the smallest animal virus examined in any detail so far, and appears to be the simplest in constitution.

The nitrogen content, 15 per cent, is indicative of a large proportion of protein, estimated as 90 per cent of the whole complex. It is not certain whether lipoid is an integral part of the virus, the largest amount extracted being 1·5 per cent. The nucleic acid is difficult to free from the virus and is relatively large in amount, about 8·7 per cent, and appears to consist wholly of the desoxypentose or thymus type (Beard, 1948).

The two strains of *equine encephalomyelitis* virus, the eastern and the western, are said to contain nucleic acid only of the ribopentose

type. If this is so, the virus is different from all other animal viruses thus far examined.

Inactivation of Viruses

There are various methods of inactivating viruses and they include the permanent loss of infectivity due to denaturation of the protein; loss of infectivity without alteration of the serological and physical properties of the virus; inhibition of infectivity.

Denaturation

This is brought about by heating and ageing, by chemicals, particularly oxidizing agents, protein precipitants or reagents causing wide changes in *p*H.

With plant viruses the conventional test consists of subjecting the virus in extracted sap to a range of temperatures for ten minutes; the temperature at which the virus loses infectivity is known as the "thermal inactivation point." However, as Bawden has pointed out, this term does not mean very much unless the conditions under which the heating is carried out are precisely defined, the *p*H of the virus solution having a marked effect on the thermal inactivation point. The "loss" of infectivity is also rather vague since different results will be obtained for different dilutions of a virus.

With some plant viruses there is general agreement among workers as to the temperature at which infectivity is lost, but with others there may be wide discrepancies. This can be explained by the relation between loss of infectivity and denaturation which differs in the various viruses. For example, in potato virus X, loss of infectivity is correlated with denaturation whereas with tomato bushy stunt virus the loss of infectivity is not necessarily closely associated with denaturation. In consequence, in addition to the temperature, other factors such as concentration of inoculum and susceptibility of test plants must be taken into account (Bawden and Pirie, 1943).

Similarly, loss of infectivity in potato virus X is correlated with a fall in serological titre but with bushy stunt virus there is no reduction in serological titre unless the virus is heated above 80°C.

The same differences are found between the behaviour of viruses when inactivated by ageing *in vitro*. Thus, after a few weeks standing at room temperature, purified preparations of potato virus X are non-infective, fail to react with antiserum or to show anisotropy of flow. On the other hand, sap, from plants infected with tomato bushy

stunt and tobacco necrosis viruses, also becomes non-infective after a few weeks but full serological activity is retained. From such sap, non-infective crystalline nucleoproteins can be isolated that are indistinguishable in their physical, chemical, and serological properties from normal virus preparations (Bawden, 1943).

Some plant viruses are inactivated by freezing, but again it depends upon the conditions under which the freezing is carried out. Thus the virus of tomato bushy stunt, in dialysed and isoelectric solution, is inactivated and coagulated by freezing; the more concentrated the solution is, the more readily it is coagulated. But at neutrality, it is more stable towards freezing than it is at its isoelectric point (Bawden and Pirie, 1943).

In the plant and in extracted sap, this virus is protected from inactivation but more prolonged freezing under those conditions may destroy infectivity without causing denaturation or loss of serological activity.

The virus of tobacco ringspot behaves in a similar way to bushy stunt virus when frozen, and tobacco necrosis virus is inactivated in the plant, if the leaves are kept dry and frozen for a week or more. When purified preparations of tobacco necrosis virus are frozen, the solution loses its characteristic opalescence and there is a reduction in infectivity and serological activity.

Bawden and Pirie (1940) have tested the effects of alkali and fifteen simple organic substances on three plant viruses, tobacco mosaic virus, tomato bushy stunt virus, and potato virus X. The viruses were all in purified form. Of these three viruses, that of bushy stunt is the most resistant to denaturation and potato virus X the least. The effects of alkali on tobacco mosaic virus are complex; gentle treatment may increase infectivity, slightly more severe treatment causes loss of infectivity but not loss of serological activity, and more severe treatment causes loss of all characteristic properties. In the case of bushy stunt virus, the range of *p*H over which inactivation occurs, is wider, and crystalline non-infective preparations can be made from alkali-treated material. Apparently similar preparations can also be made from sap which has "aged" for some months.

In the presence of alkali, sodium dodecyl sulphate destroys all the viruses tested, separating the nucleic acid from the protein.

The substances tested, besides the above, were urethane, guanidine, pyridine, picoline, lutidine, aniline, nicotine, phenol, salicylic acid, and benzoic acid. With the exception of nicotine and arginine, all

these substances, at concentrations below 4 M, inactivate the viruses in neutral solution. The two exceptions mentioned above form reversible, fibrous precipitates.

Loss of Infectivity without Alteration of Serological and Physical Properties

Certain treatments of plant viruses induce loss of infectivity without alteration of serological and physical properties. These are irradiation with ultra-violet or X-rays and treatment with formaldehyde, nitrous acid, or hydrogen peroxide.

Tobacco mosaic virus and potato virus X, when inactivated by these methods, form liquid crystals, and show anisotropy of flow in a manner similar to normal infective preparations. In the same way tomato bushy stunt virus, similarly inactivated, will form the characteristic dodecahedra when precipitated with ammonium sulphate.

Ross and Stanley (1938) claim that partial reactivation of formalized tobacco mosaic virus is possible. The virus, after being inactivated, could have a certain proportion of activity restored by dialysis at *p*H 3. For example, three preparations which had had their activity reduced to 10 per cent, 1 per cent, and 0·1 per cent were found after re-activation to possess approximately 20 per cent, 10 per cent, and 1 per cent of their original activity. Ross and Stanley consider that two simultaneous reactions occur, one reversible and the other irreversible. The fact that the addition of formaldehyde results in a simultaneous decrease in activity of amino groups and of reducing groups whilst with the removal of the formaldehyde the number of these groups is increased together with an increase of virus activity, suggests that certain of these groups play at least a partial role in the structure necessary for virus activity.

Inhibition of Infectivity

Certain substances have the power of inhibiting the activity of some viruses, especially plant viruses. Johnson (1938) showed that certain micro-organisms produced a substance which inhibits the activity of tobacco mosaic virus. Smith (1939) and Black (1939) demonstrated that there existed an inhibitor in the tissues of insects which prevented the infection of plants by tobacco mosaic and other viruses. This explained the reason why it was never possible to infect a plant by inoculating it with the juices of virus-carrying insects. Black further showed that inhibitor and virus could be separated and infectivity

restored either by filtration through collodion membranes or by high-speed centrifugation.

Other plant virus inhibitors include the sap of certain plants such as *Phytolacca* and also the enzyme trypsin. The effect of trypsin on tobacco mosaic virus is reversible and infectivity can be restored by heating. This proves that the inactivity of the virus is not due to proteolysis. There seem to be two possible explanations of the inhibition of the activity of some plant viruses by certain substances; one is that the effect is on the plant rather than the virus and the other is that, in the case of trypsin at all events, some kind of reversible complex is formed between virus and inhibitor.

In animal viruses a somewhat similar phenomenon occurs with the papilloma virus in domestic rabbits and there is an inhibitor associated with the chicken tumour I virus which Claude (1939) considers to be a protein.

Absorption Spectra

Ultra-violet light absorption spectrum measurements have been made on purified preparations of several viruses and they show in general an absorption maximum at about 2600 Å and a minimum about 2500 Å. This is what would be expected of substances containing nucleic acid, which is known to absorb strongly at 2600 Å.

The amount of the absorption is related to the nucleic acid content, for tobacco ringspot virus with a nucleic acid content of about 40 per cent absorbs most strongly, and bushy stunt, tobacco necrosis, chicken tumour I, potato virus X, and tobacco mosaic virus preparations with nucleic acid contents of about 20 to about 5 per cent absorb proportionally less (Stanley, 1940[b]).

Size and Shape of Viruses

There are six main methods of measuring the size of virus particles; these are by (1) ultra-violet light and electron microscopy, (2) X-ray diffraction, (3) sedimentation and diffusion, (4) ultra-filtration, (5) radiation inactivation, (6) measuring the light scattered by solutions of viruses.

The most direct method of finding the size of a virus is by ultra-violet light or the electron microscope. The great merit of the use of the electron microscope is that the measurement of size is direct and is not subject to error due to the necessity of interpreting the measurements by theoretical formulae.

The X-ray diffraction method is only applicable to viruses obtainable in a crystalline or semi-crystalline form. In a tomato bushy stunt virus crystal, or a tobacco mosaic virus gel, the virus particles are arranged in a regular array or pattern. This array serves as a diffraction grating to a beam of X-rays, and measurement of the angles at which the X-rays of known wave-length are diffracted enables the plan of the pattern and the repeat interval to be established. Thus, in the case of wet crystals of bushy stunt virus, it has been found that the pattern is a body-centred cubic lattice of edge 394 Å. In other words, if one imagines the crystal to be built up out of cubes of this size, then at the centre of each cube and at every corner there is either a virus particle, or an identical group of virus particles.

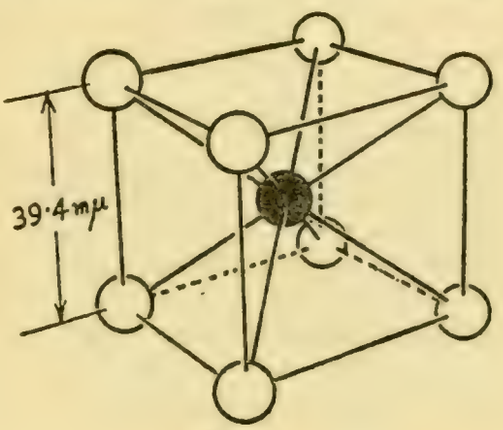


FIG. 1

There will thus be two particles, or groups, per cube of edge 39·4 mμ, and knowing the density of the crystal, the molecular weight of the particle, or group, can be calculated (*see* Fig. 1). Calculated by this method the particle size of the tomato bushy stunt virus is 34 mμ when wet. When the crystal is dried it shrinks and the size of the unit cell also decreases to 31·6 mμ, the dry virus particle being about 27·6 mμ in diameter.

In the method of determining size by sedimentation and diffusion, the experiments consist in determining S_{20} and D_{20}, the sedimentation and diffusion constants respectively. The *sedimentation constant* is determined by measuring the rate at which particles sediment through water in a gravitational field, but as this rate would be very slow in the earth's gravitational field, it is necessary to put the solution into an ultracentrifuge, the fields used being 2000–18,000 × *g*. The *diffusion constant* is a measure of the rate of movement of the virus particles through water, impelled by Brownian movement. For a spherical, non-hydrated virus, the partial specific volume of which is known, measurement of either S_{20} or D_{20} alone gives the size of the virus. With both together, however, a much better estimate of the size is obtained.

The method of determining the size of a virus by filtration through collodion membranes of graded porosity depends upon (*a*) determining the pore size of each membrane used in terms of a conventional measure known as the "average pore diameter" (A.P.D.), (*b*) relating

for the type of membrane used the A.P.D. to the size of the particle just stopped by the membrane. This is the method used by Elford (1931).

TABLE III

THE PARTICLE SIZE OF SOME REPRESENTATIVE VIRUSES AND PROTEINS

Virus	*Size* mμ	*Method of Measurement*
Bacteriophage S 13	8–12	ultra-filtration
Bacteriophage staph. (Northrop)	550–670	centrifugation
Equine encephalomyelitis virus	32–39	centrifugation
Foot-and-mouth disease virus	8–12	ultra-filtration
Gipsy moth virus (*L. dispar*)	415 × 160 (virus bundles)	electron microscope
Influenza A virus	80–120	ultra-filtration
Potato virus X	9·8 × 433	centrifugation
Psittacosis virus	280 × 380	visible light microscope
Southern bean mosaic virus	31	sedimentation, diffusion, and viscosity measurements
Tobacco mosaic virus	15 × 275	electron microscope
One of the tobacco necrosis viruses	20	electron microscope
Tomato bushy stunt virus	26	electron microscope
Turnip yellow mosaic virus	19·5	electron microscope
Vaccinia	240 × 170	electron microscope
PROTEINS		
Helix haemocyanin	31	centrifugation and electron microscope
Horse methaemoglobin	5·7	X-ray diffraction

The recent method of radiation inactivation is based on the fact that, when a virus is inactivated by ionizing radiation (i.e. X-rays or a radio-active radiation, but not ultra-violet light), it is possible to calculate from the amount of inactivation produced by known doses of radiation what may be called the radio-sensitive volume of the

TABLE IV

COMPONENT CONSTITUTION OF SOME ANIMAL VIRUSES AND T_2 BACTERIOPHAGE

	Whole Complex				Lipoid				Nonlipoid				
	C	N	P	Carbo-hydrate	Total	Phos-pholi-poid	Choles-terol	Neutr. fat	Total	Protein	Carbo-hydrate	Nucleic Acid	
												INA	RNA
Broth bacteriophage .	42·0	13·5	4·84	13·6	2·6	0·0	0·0	2·6	97·4	50·6	13·1	40·3	6·6
Synthetic medium bacteriophage . .	42·3	13·3	5·22	11·7	1·8	0·0	0·0	1·8	98·2	52·4	11·2	44·6	1·3
Vaccinia . .	33·7	15·3	0·57	2·8	5·7	2·2	1·4	2·2	94·0	89·0	2·8	5·6	
Papilloma . .	49·6	15·0	0·94	6·5	1·5				98·5	90·0		8·7	
Equine encephalomyelitis (Eastern strain)	62·2	7·7	2·2	4·0	54·1	35·0	13·8	9·6	53·0	49·1	7·2		4·4
Influenza A (PRS strain) . .	53·2	10·0	0·97	12·5	23·4	11·3	7·0	5·1	77·0	65·0	7·3	1·5	?
Influenza B (Lee strain) . .	52·7	9·7	0·94	13·1	22·4	11·2	3·7	7·2	76·4	63·6	9·4	1·2	?
Swine influenza .	51·4	9·0	0·87	10·0	24·0	10·7	5·7	7·7	77·6	67·6	10·0	+	?
Broth med. bact. .	49·1	13·2	2·72	12·5	7·75	7·75	0·0	0·0	92·3	67·9	12·5	5·2	19·1
Synthetic medium bacterium . .	49·0	13·2	2·66	11·6	9·11	9·11	0·0	0·0	90·9	67·7	11·6	2·4	20·9

All values are percentage dry weight of the whole complex

(*From* J. W. Beard, *Physiol. Review*)

virus, i.e. the volume of that part of the virus within which energy must be absorbed from the radiation for inactivation to occur. Absorption is a highly localized phenomenon, and is sufficiently energetic for it to be tolerably certain that when energy absorption, i.e. ionization, occurs in a particular atom, the molecule or radicle of which that atom is a part suffers chemical change. In a large virus like that of vaccinia, the radio-sensitive volume is only a very small fraction of the volume of the virus. On the other hand, a virus which crystallizes is presumably a molecular species and it seems legitimate to conclude that the radio-sensitive volume will be identical with the volume of one molecule of the virus. For further information on these methods of measurement of virus-particle size, the reader is referred to a monograph by Markham, Smith, and Lea (1942).

A more recent method of measurement has been described by Oster (1947); experiments have shown that the size and shape of high polymeric molecules, colloidal particles, and viruses can be determined by measuring the light scattered by solutions of these substances at different angles to the incident light.

CHAPTER VII

ELECTRON MICROSCOPY OF VIRUSES

SINCE the limit of resolution of the optical microscope in visible light is about 200 mμ, an object must be at least 250 mμ in diameter to be properly resolved. This is far larger than most animal, and so far as we know, all plant viruses.

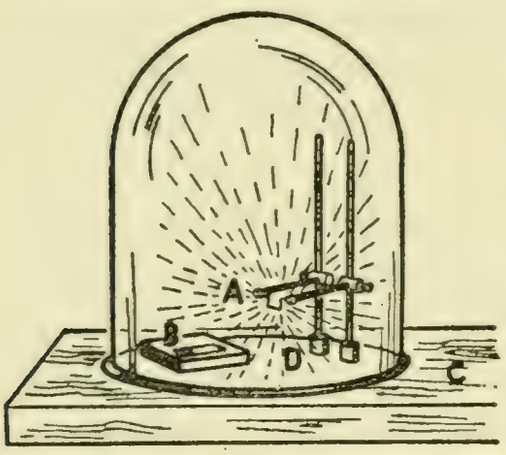
The wave-length of ultra-violet light which can be used with a quartz objective is not much below 2500 Å and so only the larger viruses of 100 mμ and above can be measured by this means. For the examination of the small viruses, therefore, it is clear that a radiation with a wave-length shorter than that of light is necessary. In the electron microscope the illuminating radiation is provided in the form of a swiftly-moving beam of electrons which corresponds to the beam of light in the optical microscope.

The "lenses" of the electron microscope cannot be made of glass which is opaque to the electron beam but electric or magnetic "lenses" are used instead, to focus the electrons. Because electrons are scattered by gas molecules it is necessary for the electron optical paths to be evacuated and the specimen must be dry. The specimen is mounted on a thin film, usually of collodion, supported on a fine metal grid.

The focal length of a magnetic lens is a function of the current through the lens coil; hence the magnification is controlled by varying the current through the magnetic lenses. It is necessary, of course, for the current to be constant to avoid blurring of the image and to obtain high resolution. The source of electrons is a hot filament and the beam of electrons is focused on to the object by the first, or condenser lens, coil. Electrons from the central portion of the intermediate image formed by the objective or second magnetic lens coil are re-imaged by the third or projection lens coil and the final highly magnified image of the specimen may be viewed directly on a fluorescent screen or photographed by means of a film sensitive to electrons (Stanley, 1943) (Plate XI).

A great improvement in the technique of electron micrography has been described by Williams and Wyckoff (1946). This is known as metallic shadow-casting, and the method is, briefly, as follows: The sample is coated with a semi-transparent layer of metal deposited

obliquely by evaporation in a vacuum. To do this the collodion film bearing the virus or other particles is put on an ordinary glass microscope slide. The slide is then fixed inside a suitable container, such as a bell jar made of thick glass, at an oblique angle to a filament charged with a weighed amount of the metal to be evaporated. The air is then evacuated and, when the vacuum is complete, the metal filament is heated to incandescence by an electric current which volatilizes the metal and deposits it obliquely on the virus particles (*see* Fig. 2). Various metals have been used, particularly gold, chromium, nickel, and uranium, but gold seems to be the one most suitable for small virus particles though it is prone to form artefacts when viewed in the electron microscope (Mandle, 1947). This gives a three-dimensional effect to the image so that the heights or thicknesses of discrete objects which are not wholly flat can be estimated from the lengths of the shadows they cast. Shadow-casting brings out in great relief the surface contours of the specimen by enhancing the scattering power of the entire preparation. The type of detail brought out by shadowing can be varied by altering the thickness of the evaporated layer. In addition it makes clearly evident objects so small or thin that by themselves they would not produce a minimum contrast against the background of the rest of the specimen (Williams and Wyckoff, 1946). Photographs of viruses taken by the shadow-casting method are illustrated in Plates XII, XIII, and XIV. One result then of the application of electron microscopy to the study of viruses has been to increase greatly our knowledge of the morphology of virus particles.



(*After Wyckoff*)

FIG. 2. A DRAWING TO OUTLINE THE EXPERIMENTAL PROCEDURE FOR SHADOW CASTING

Metal evaporated in vacuum from a hot filament *A* deposits obliquely upon the specimen *B*

But the electron microscope does more than define the shapes and sizes of virus particles; it can also reveal the apparent structure of the larger viruses.

Under dark ground illumination with the optical microscope, vaccinia virus appears as a spherical particle, but when photographed by the electron microscope the particles are seen to be brick-shaped with rounded corners. Occasionally a particle is photographed standing on end and it appears to be circular in cross-section. Dense

patches can be seen in the virus particles indicating some kind of internal organization. This central "nucleus" of dense material has been investigated by McFarlane and Dawson (1948) after special treatments. Two important chemical reactions of viruses which are open to examination under the electron microscope are their inactivation by various methods and their neutralization by specific antisera or, to put it another way, the antigen-antibody reaction. Not much work has been done on the microscopy of inactivation though it is known that the virus particles do not appear to have changed much after treatment with formaldehyde. The particles of tobacco mosaic virus have been photographed after supersonic radiation and have been shown to be split up into smaller non-infectious units.

Anderson and Stanley (1941) have used the electron microscope to study the reaction between tobacco mosaic virus and its antiserum. Photographs taken of the purified virus alone showed it to consist of particles about 280 mμ long and about 15 mμ wide. Micrographs of a mixture of this virus and normal rabbit serum showed virus particles of normal size and indicated little or no adsorption of the serum particles on to the virus particles.

Similarly, no adsorption took place between the tobacco mosaic particles and antisera from rabbits prepared against the viruses of tomato bushy stunt, potato virus X, and tobacco ringspot. However, when its specific antiserum is added to the tobacco mosaic virus, a different state of affairs is revealed under the electron microscope. When dried on a collodion film an hour after mixing, photographs show particles about 300 mμ long by 60 mμ wide with a characteristic fuzzy profile. If the mixture is applied to the collodion film several hours after mixing, an irregular framework of thickened antigen (virus) particles may be seen. It is suggested that the fuzzy profile of the virus particles is due to an adsorbed layer of asymmetrical molecules from the serum, arranged along the length of the particle with their long axes at right angles to the axis of the virus particle whilst the irregular framework, formed after several hours, may possibly be the antigen-antiserum precipitate.

A similar study has been made of the antigen-antiserum reactions of two viruses, those of tomato bushy stunt and southern bean mosaic which have spherical particles, but using the metal shadow-casting technique in a pure suspension and at sufficient concentration, those viruses show a pronounced tendency to associate in regular crystal-like arrays. When mixed with their specific antisera, however, the particles

appear aggregated without the same regularity into microclumps in which the particle separations seem to be about twice their normal value (Black, Price, and Wyckoff, 1946).

When a suspension of typhus rickettsiae is extracted with ether, a "soluble" antigen may be liberated which has many of the immunological properties of the rickettsiae themselves. This antigen, whose active principle will pass the usual bacteria-proof filters, is highly active in the complement fixation and precipitation reactions. Electron micrographs of a concentrated suspension of typhus or Q-fever rickettsiae show them to be enmeshed in a thin membranous material suggestive of bacterial capsules. Extraction with ether at room temperature does not alter the appearance of the rickettsiae themselves, but it does profoundly affect their capsules which are broken up and, in a sense, emulsified. It appears, therefore, that the "soluble" antigen of typhus and probably of other rickettsiae consists of submicroscopic particles of a capsular substance (Shepard and Wyckoff, 1946).

Another interesting and important application of the electron microscope is the study of induced changes such as the action of the bacterial viruses upon bacteria. Such a study should yield information as to how viruses develop and multiply within their host cells, and bacteria, as Wyckoff (1948) has pointed out, are very suitable for this purpose as they are small enough to be partly transparent in the electron microscope.

Wyckoff (1947[c] and 1948) has studied the lysis of *E. coli* by the T strains of bacteriophages. The protoplasm of the lysed bacteria has a characteristic fine structure with an extraordinary degree of regularity. It consists of elements, about the size of bacterial virus particles, which usually appear as concavities and are frequently lined up in rows or regular networks throughout the protoplasmic mass. Wyckoff has also recently shown that filaments, as well as spheres, are present in purified influenza virus suspensions and that an intimate relation seems to exist between the two forms.

If plant viruses multiply by binary fission, then it should be possible sooner or later to photograph the virus particles in the act of dividing.

Examples of such fission are rarely seen in the routine photography of purified virus solutions but if it were possible to photograph the virus inside the living cell, it may be that dividing particles of viruses would be more frequently seen.

It is a characteristic of viruses that they mutate and some electron

microscope studies have been made of mutations of two well-known plant viruses with rod-shaped particles, that of tobacco mosaic and potato virus X. The resulting photographs showed no perceptible difference in the appearance or length of the particles of these two viruses and their strains (Takahashi and Rawlins, 1946 and 1947).

The electron microscopy of the crystallizable viruses now offers a direct approach to questions of how crystals are built up from their molecular units and studies from this aspect have been carried out on several plant viruses, particularly southern bean mosaic virus, tomato bushy stunt virus, one of the tobacco necrosis viruses, and turnip yellow mosaic virus.

Micrographs of the two first-named viruses from purified solutions showed a tendency towards a regular arrangement of the elementary particles or virus molecules. Different types of field were seen in electron micrographs of the bushy stunt virus. In one, the elementary particles appeared as well-defined spheres that packed closely together in a regular array. In other parts of the preparation the particles were ill-defined in clumps and masses.

Particles of southern bean mosaic virus are similar both in size and shape to those of bushy stunt virus. Regions of regular particle arrangement, however, are far more numerous in the bean mosaic preparations, and there is a pronounced tendency for the regular layers of particles of this virus to be stacked on top of one another in a three-dimensional crystalline array (Price, Williams, and Wyckoff, 1946).

One of the tobacco necrosis viruses crystallizes in thin, flat plates which are very suitable for electron micrography. Photographs of single virus crystals showed very plainly the molecular arrangement over the face of the crystal. Later attempts recorded the regular molecular distribution over several faces of crystals of this virus, thereby demonstrating their truly crystalline structure. The upper photograph of Plate XVI is a micrograph of a single tobacco necrosis virus crystal showing the close-packed molecular net on the pyramidal (inclined) faces and the square net on the horizontal face (Markham, Smith, and Wyckoff, 1947, 1948).

The structure of turnip yellow mosaic virus crystals has been examined in the electron microscope and micrographs have been taken (by transmission) of the virus mounted on beryllium films. Some of these show a number of micro-crystals having the appearance of a net formed by hexagonal rings. Each ring has an electron-transparent

hole in the centre, of such a size that the rings appear larger than the size of the individual virus particles. From the photographic density of the images it may be estimated that the crystalline fragments are only a few molecules thick in the direction of the electron beam. Evaluation of the angles and particle separation in different directions shows that it is a diamond-type lattice, giving a size for the dry particle of the virus of 19·5 mμ on the assumption that it is rigid and undergoes no distortion on entering the lattice (Cosslett and Markham, 1948).

In concluding this short account of the electron microscopy of viruses we may appropriately quote these words of Wyckoff (1946)—

> We span that range of organized matter which extends from the animate to the lifeless and which must be understood as a basis of what we intuitively mean by "living"; at the same time we acquire the ability to "see" the larger of the molecules that are the basic units of chemistry. It rarely happens that any new experimental technique allows as direct an approach to the problems of a single science as the electron microscope thus gives to the fundamentals of both chemistry and biology.

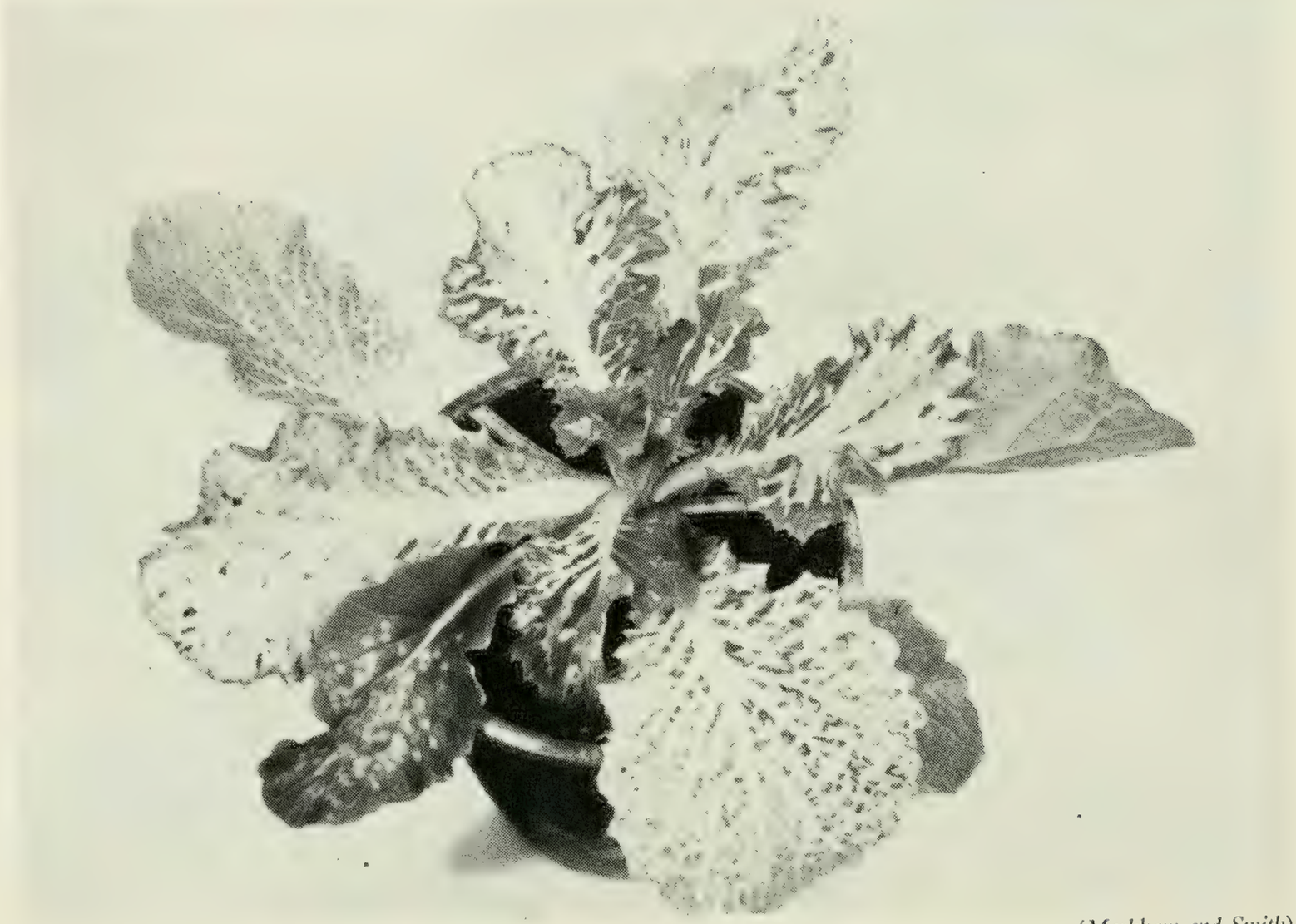
Plate I

Stylets of a Sap-sucking Insect, an Aphis, in Position in the Plant Tissue

Note that the vascular bundle is being tapped.

Photomicrograph of a Section through the Oesophagus and Foregut of an Aphis, showing the Valve (marked by arrow) which Prevents Regurgitation

(*Markham and Smith*)



(*Markham and Smith*)

PLANT OF CHINESE CABBAGE INFECTED WITH TURNIP YELLOW MOSAIC

Note the bright mosaic mottling.

Plate III

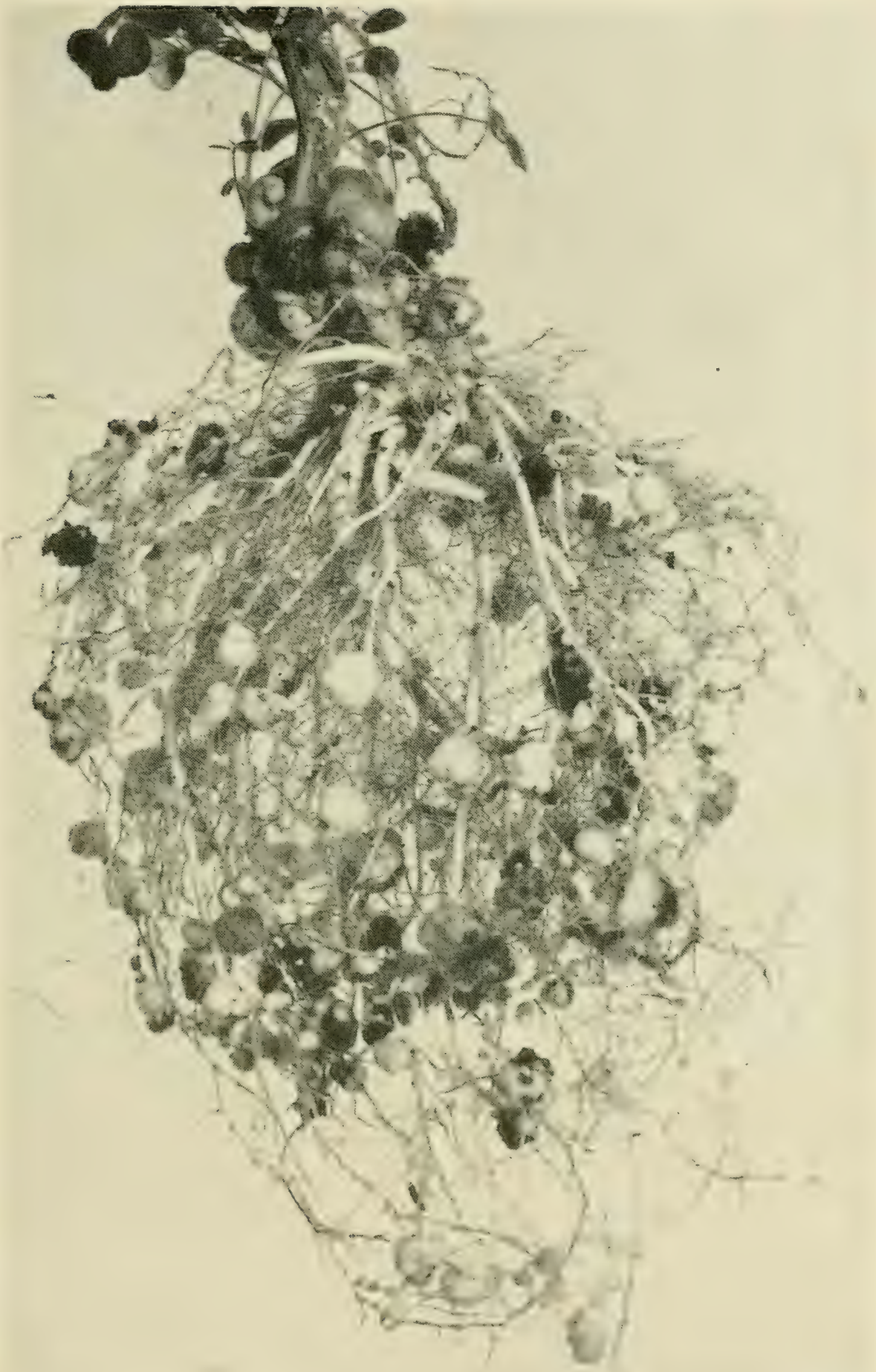
(*Markham and Smith*)

Leaf of a Tobacco Plant Infected with a "Ringspot" Virus

LEAF OF A TOBACCO PLANT INFECTED WITH THE VEIN-DISTORTING VIRUS

Note how the leaf is twisted on itself.

Plate V

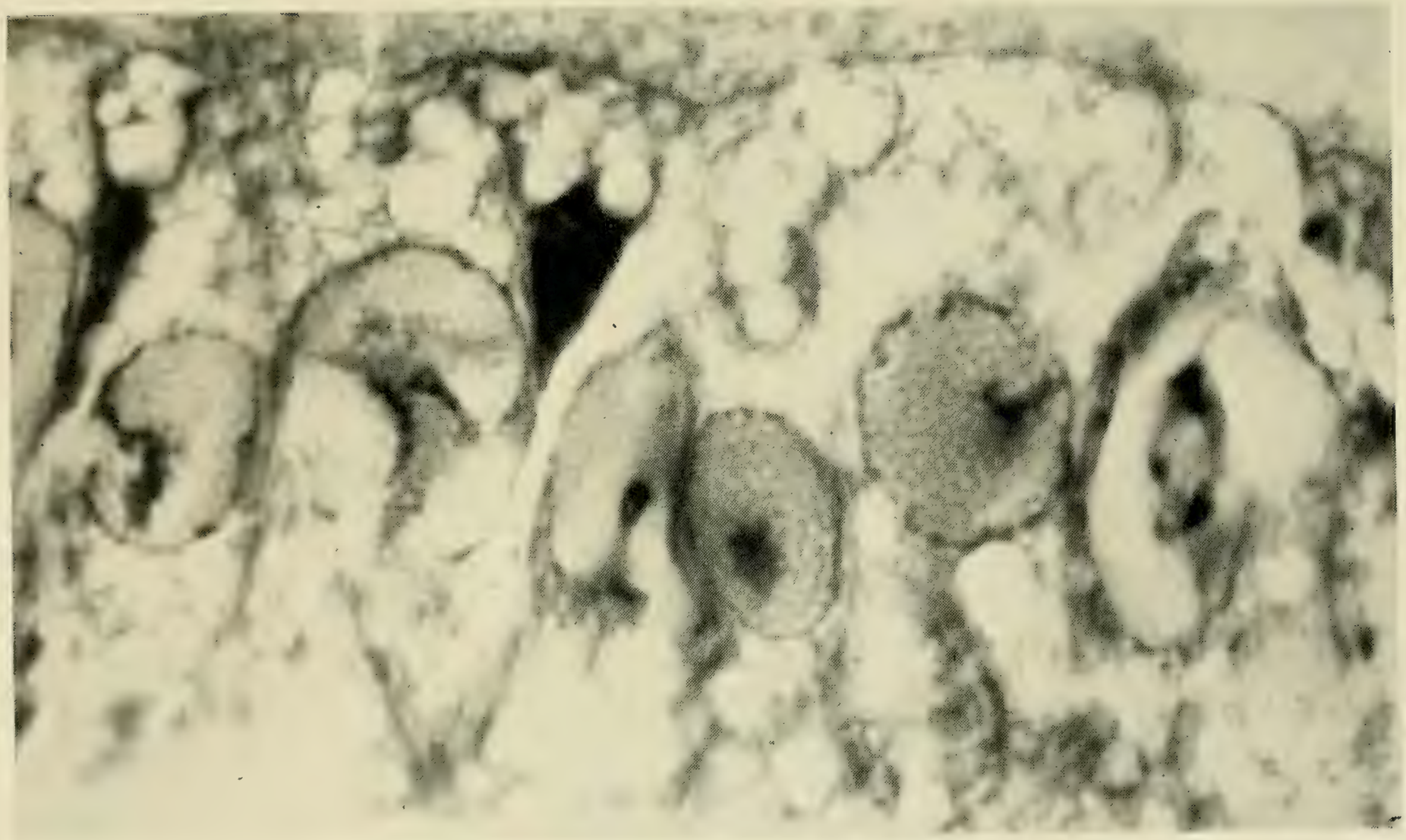


(*After Black*)

Leguminous Plant Infected with the
Wound Tumour Virus

CATERPILLAR OF THE CURRANT MOTH (*Abraxas grossulariata*)
AFFECTED WITH A POLYHEDRAL VIRUS DISEASE

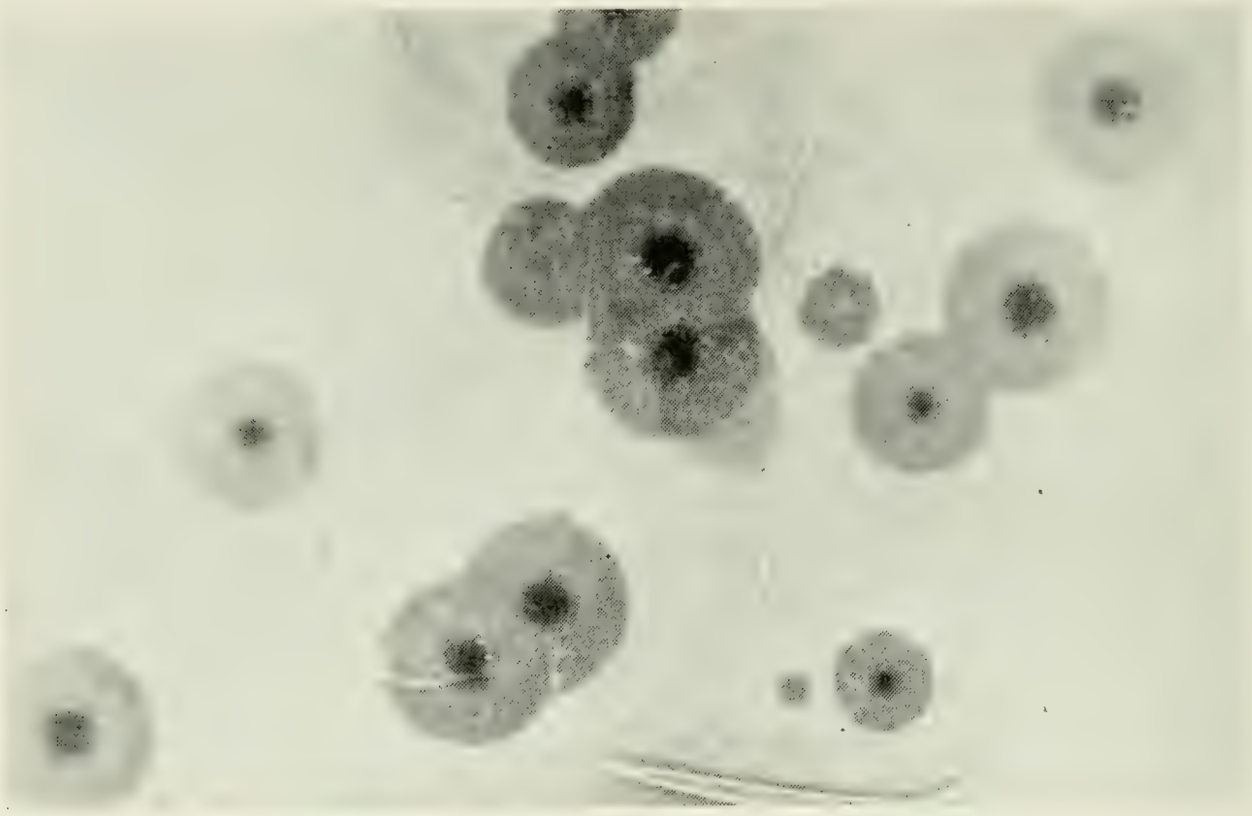
Note how the skin has burst and liberated masses of polyhedral bodies.



PHOTOMICROGRAPH OF A SECTION THROUGH A CATERPILLAR OF THE CURRANT MOTH (*A. grossulariata*) AFFECTED WITH A POLYHEDRAL VIRUS DISEASE

Note the enormously enlarged cell nuclei packed with polyhedral bodies.

Plate VIII



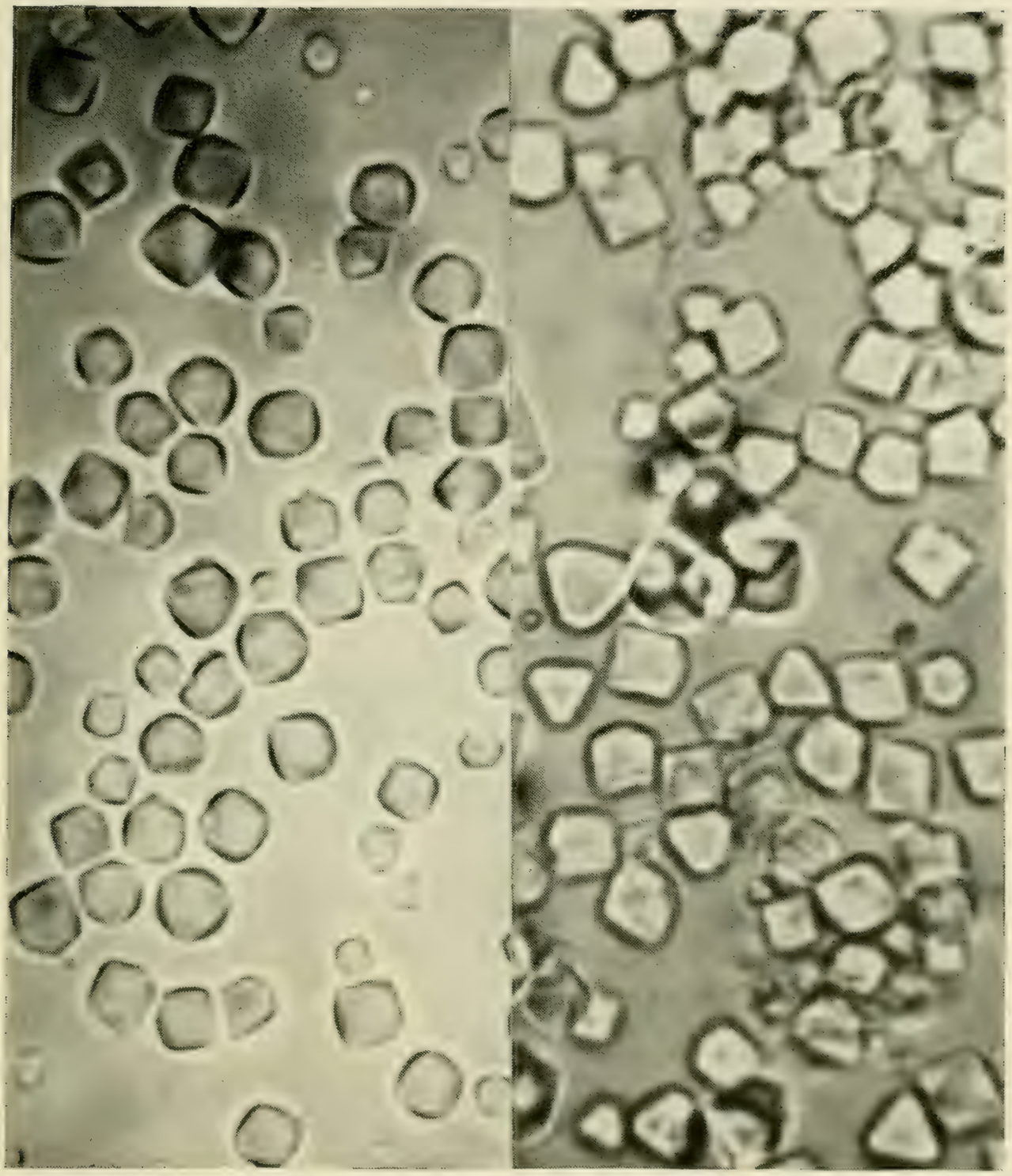
Photograph of a Culture of Bacteria Attacked by a Bacterial Virus

The dark circles are foci of lysis, or destruction, of the bacteria with complete lysis in the centre.

(*Markham and Smith*)

Octahedral Crystals of the Turnip Yellow Mosaic Virus

PLATE IX



(*Markham and Smith*)

CRYSTALS OF THE WHOLE VIRUS OF TURNIP YELLOW MOSAIC (*right*) COMPARED WITH CRYSTALS OF THE NUCLEIC-ACID FREE COMPONENT WHICH IS NOT INFECTIOUS

Plate X

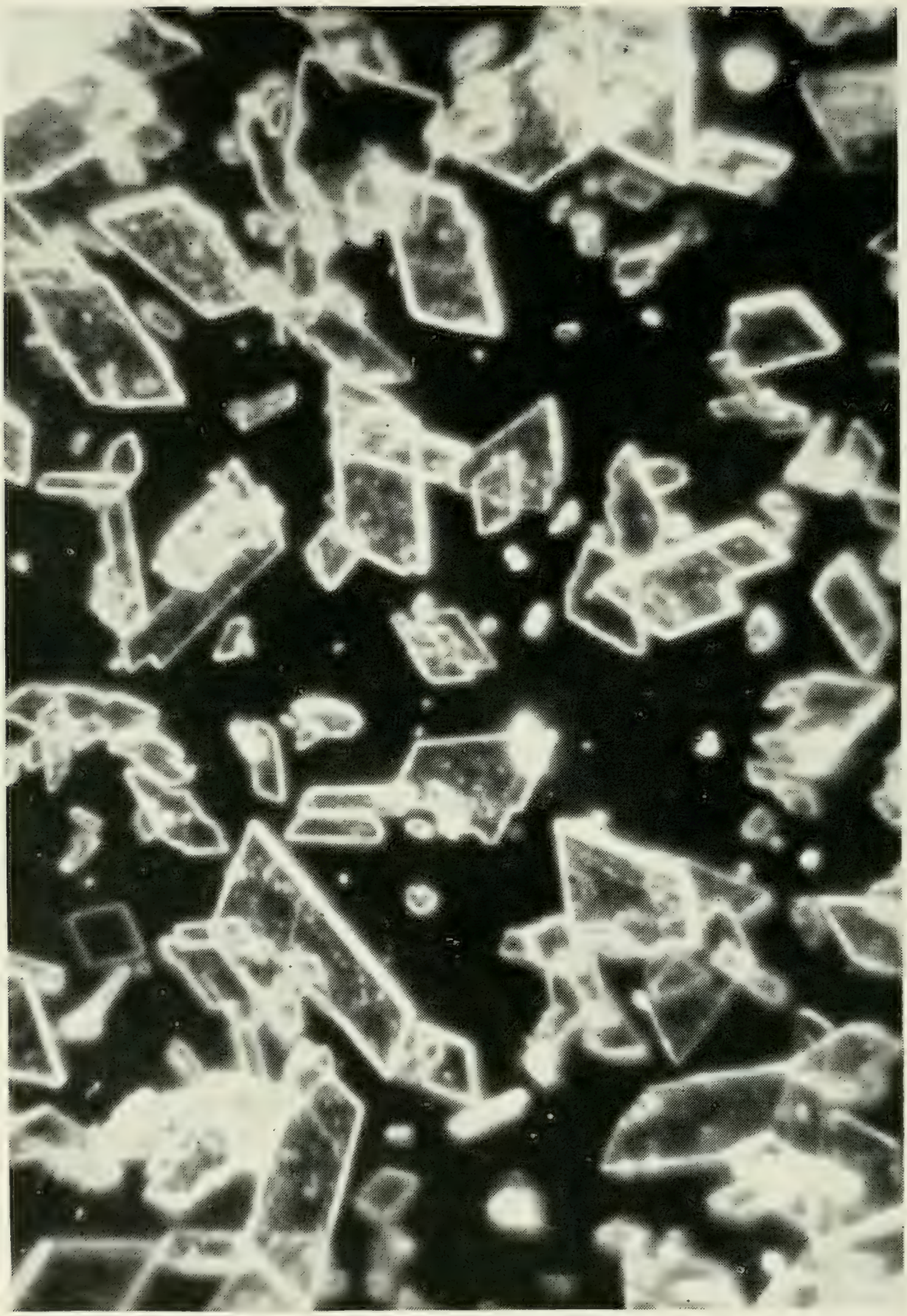
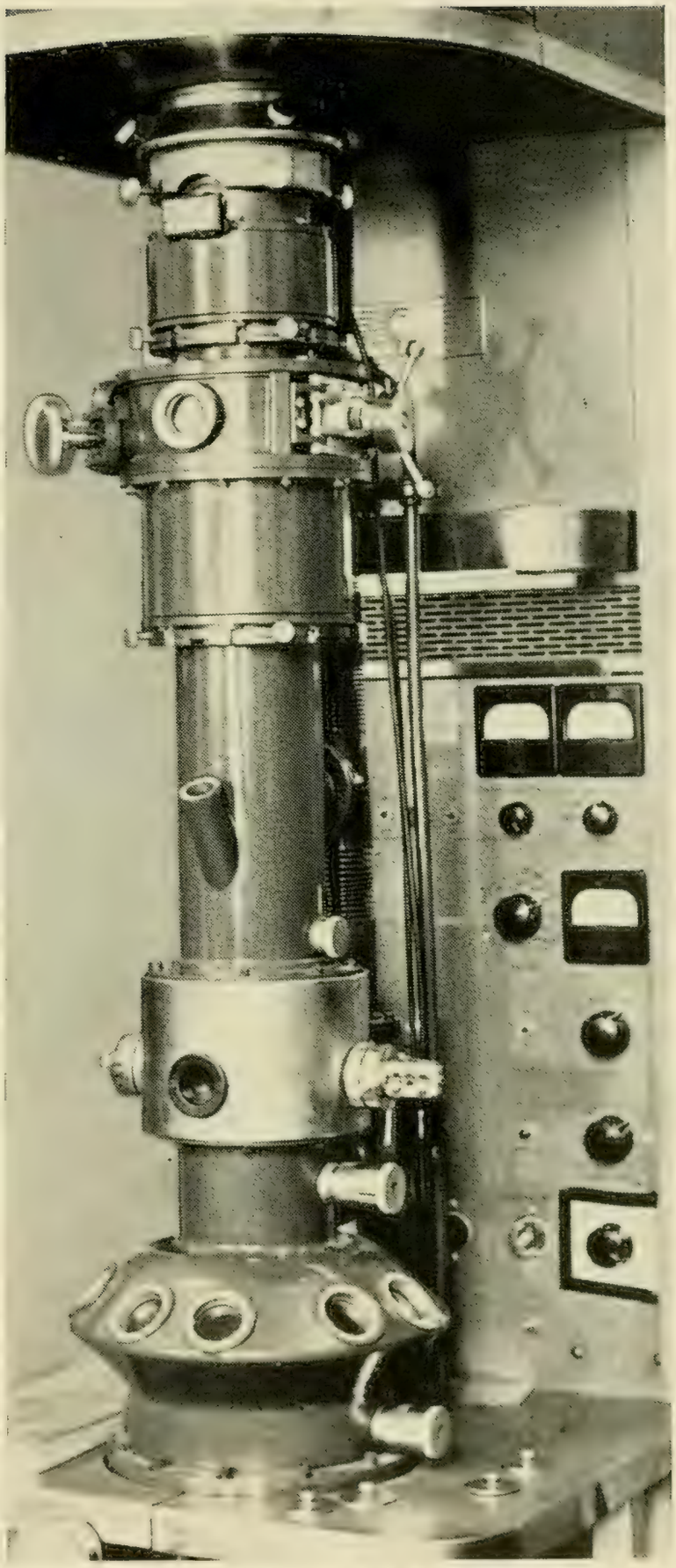


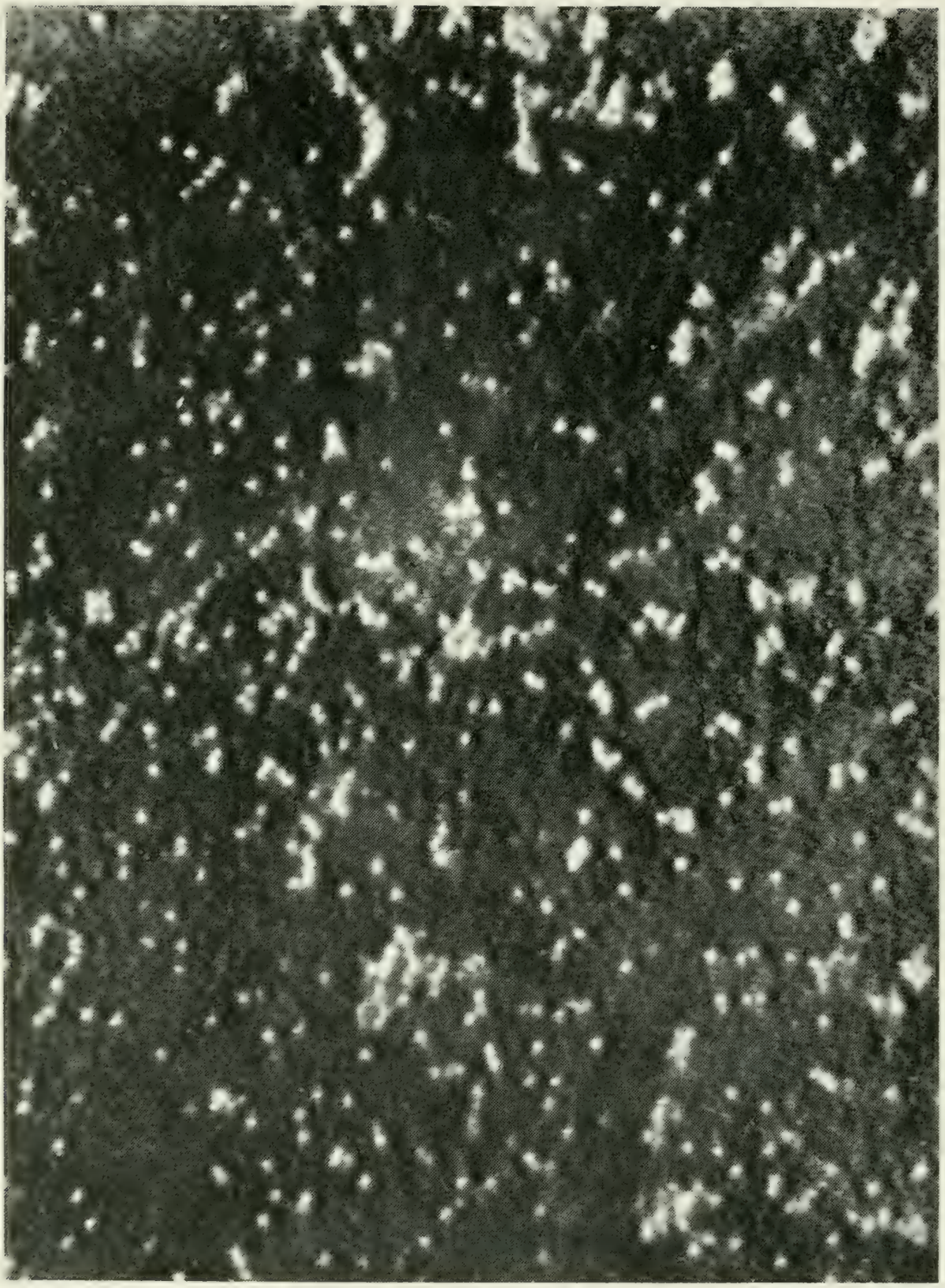
Plate-like Crystals of a Tobacco Necrosis Virus, Photographed under Dark Ground Illumination

Plate XI



The Electron Microscope (R.C.A.)

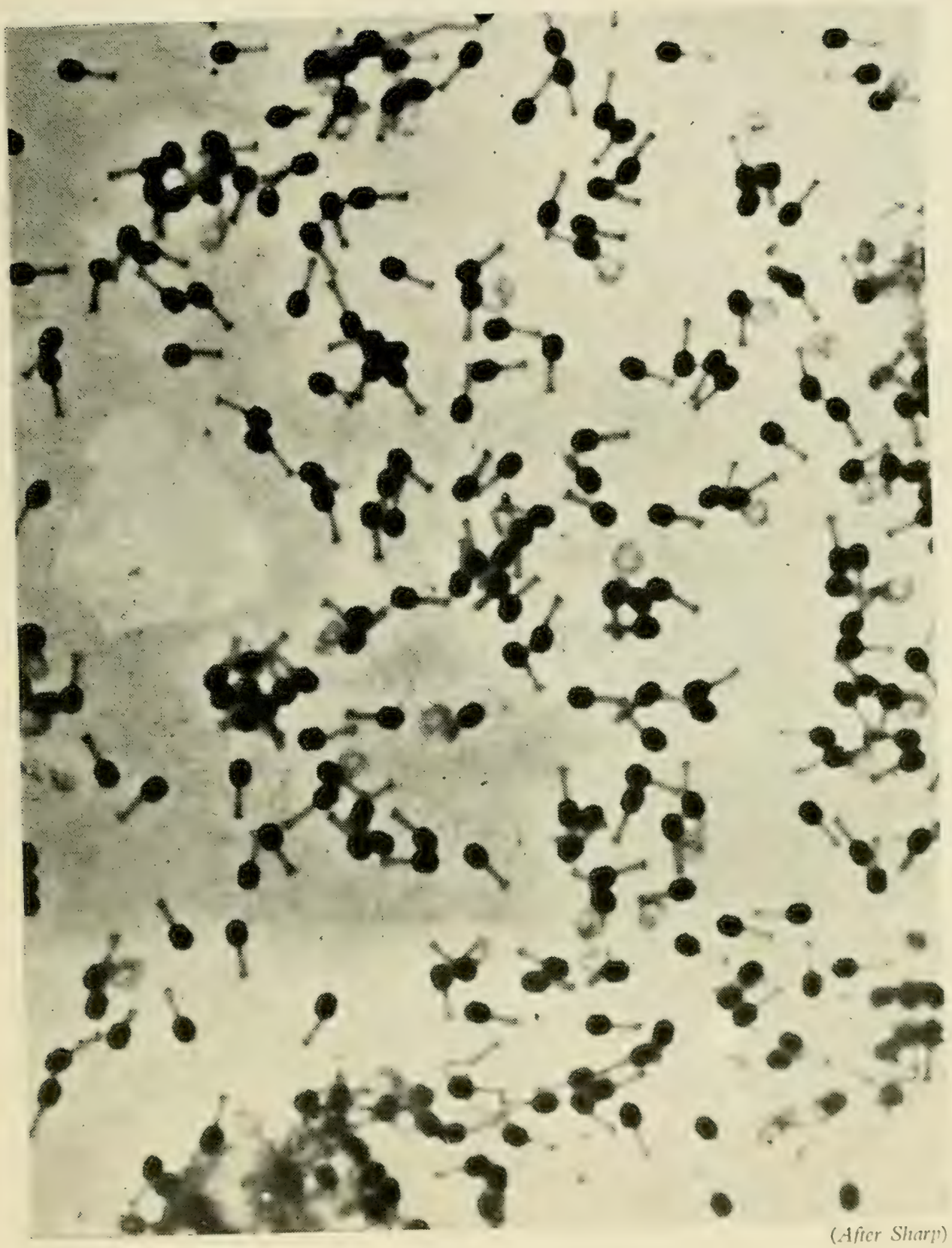
PLATE XII



(*Markham and Smith*)

AN ELECTRON MICROGRAPH OF THE TURNIP YELLOW MOSAIC VIRUS, PHOTOGRAPHED BY THE GOLD-SHADOW TECHNIQUE (× *c.* 65,000)

PLATE XIII

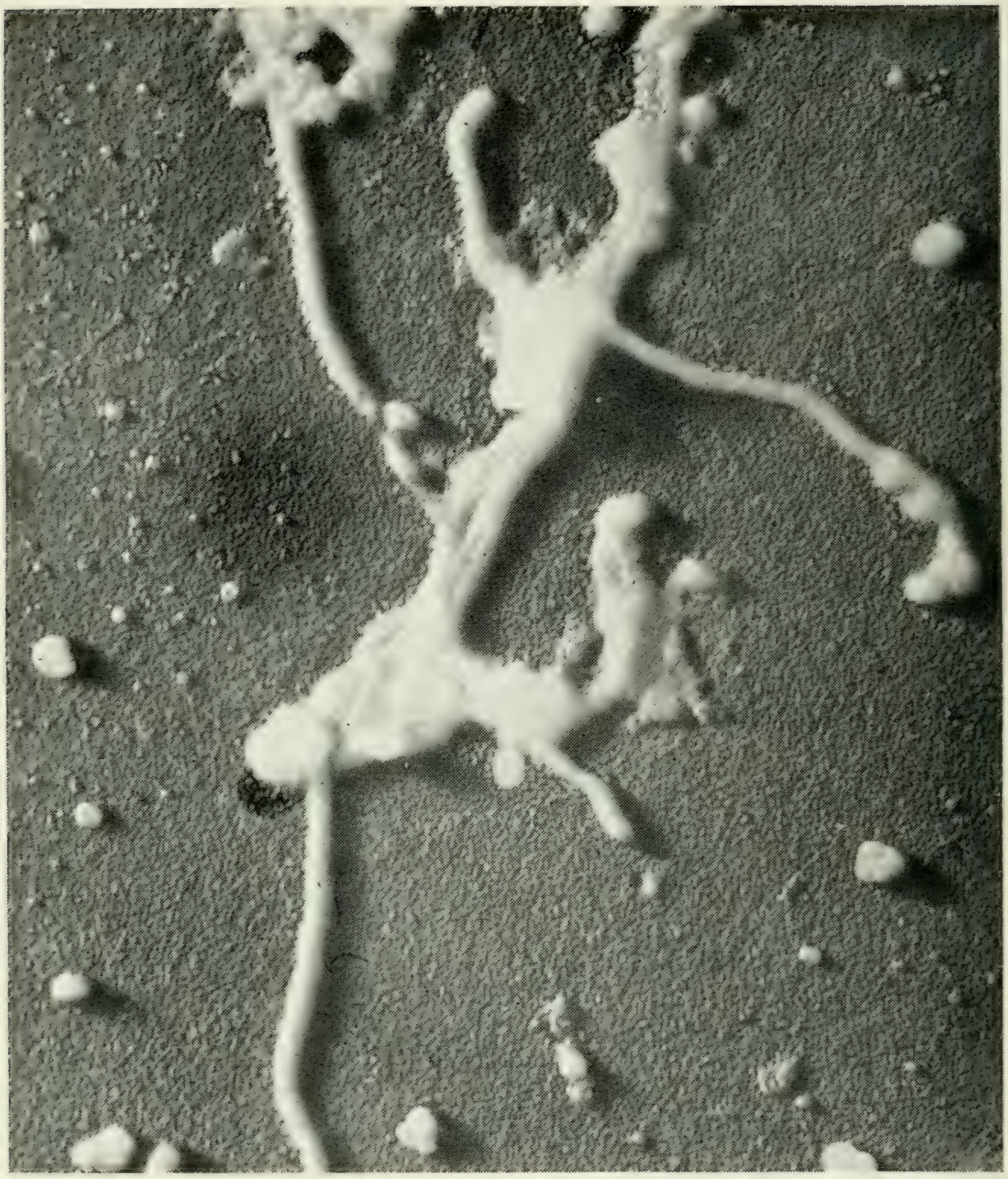


(*After Sharp*)

AN ELECTRON MICROGRAPH OF A BACTERIAL VIRUS

Note the "head" and the "tail."

Plate XIV

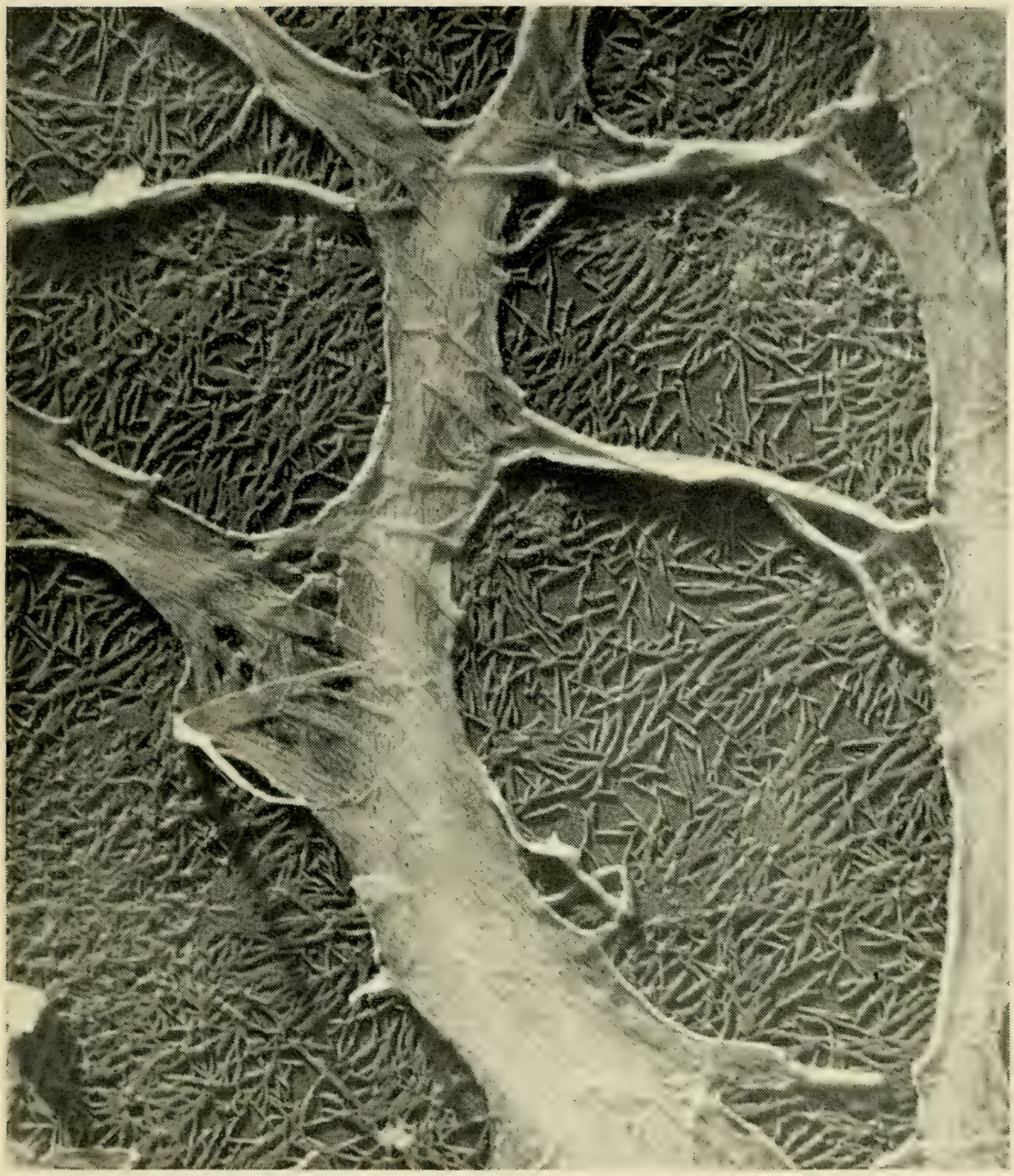


(*After Mosley and Wyckoff*, Nature, 1946)

An Electron Micrograph of the Weiss Strain of Influenza Virus (× *c.* 21,000)

Note the elongated forms associated with the spherical particles.

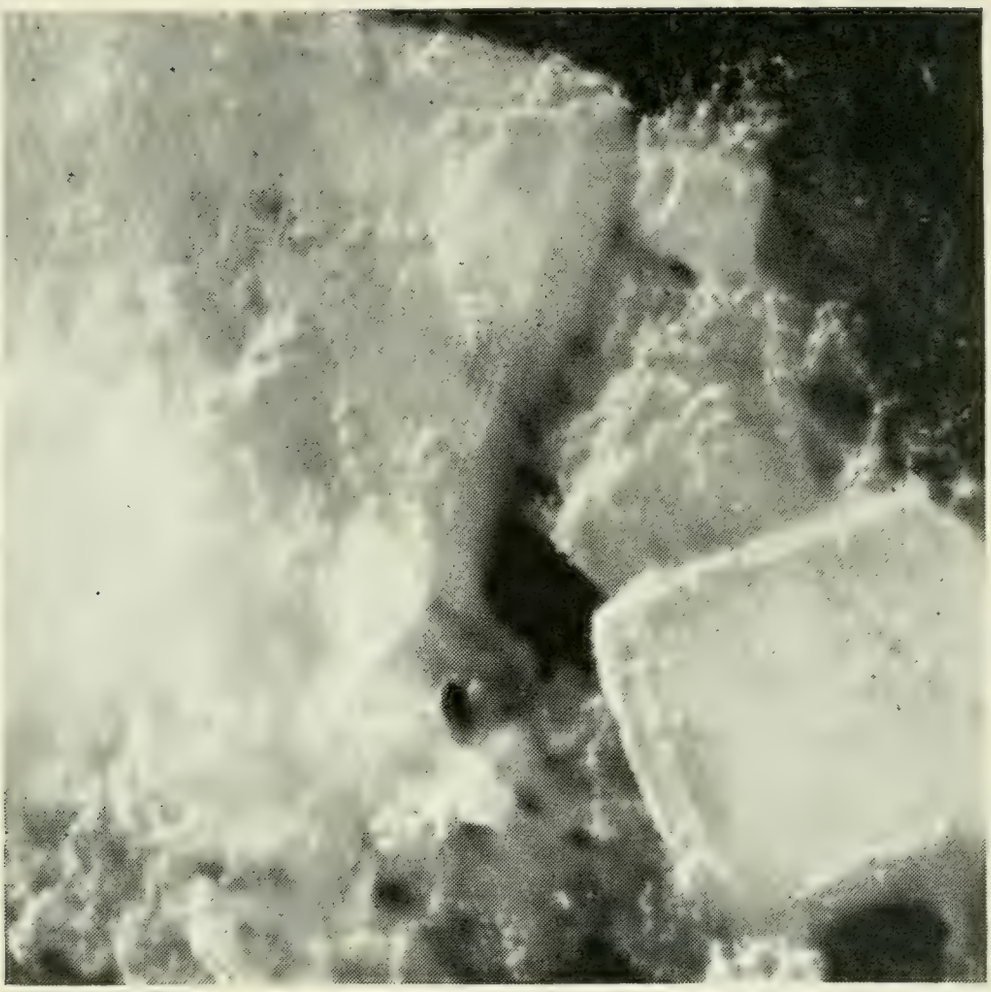
Plate XV



(*After Wyckoff*)

An Electron Micrograph of a Frozen-dried Solution of Tobacco Mosaic Virus (× *c.* 13,000)

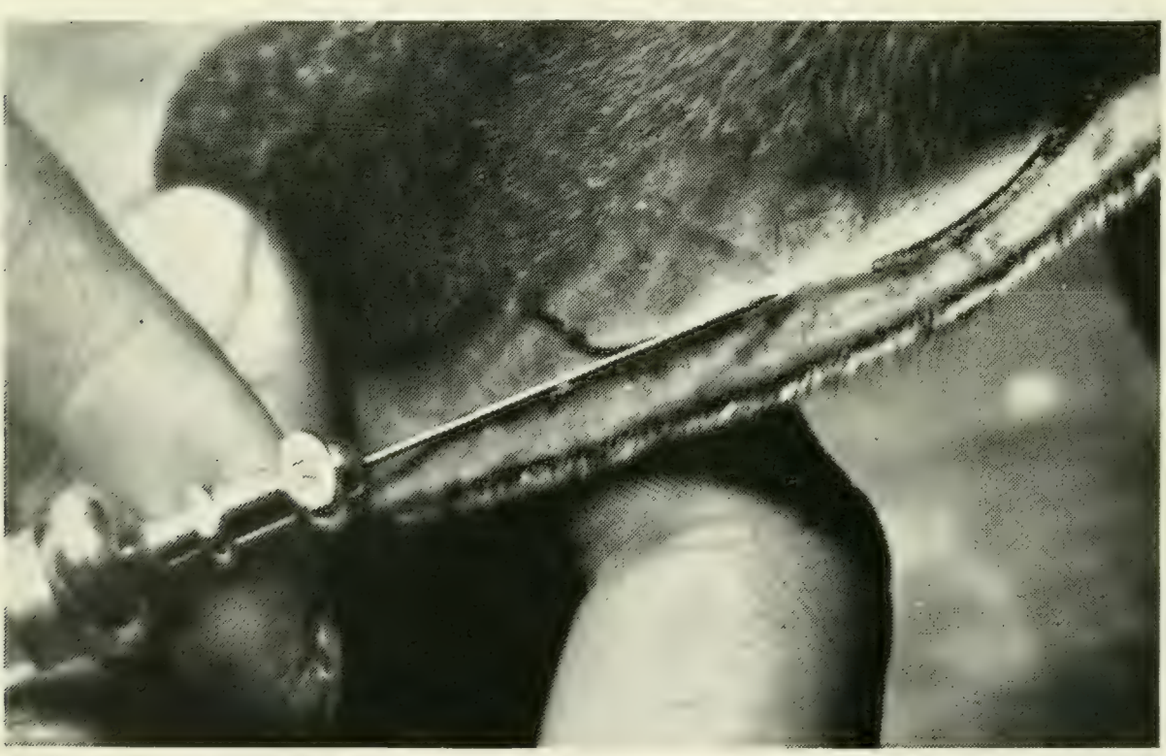
Plate XVI



(*Markham, Smith, and Wyckoff*)

Individual Crystals of Tobacco Necrosis Virus
Photographed on the Electron Microscope

Note the regular arrangement of the virus molecules on the crystal face.



(*Markham, Matthews, and Smith*)

How to Inoculate a Rabbit with a Plant Virus
to Produce the Antiserum

The vein to be used is outlined in Indian ink. The injection is being done by a left-handed person.

CHAPTER VIII

SEROLOGY OF VIRUSES

It is manifestly impossible to deal adequately with a large and highly technical subject like the serology of viruses in one chapter, and no attempt is made to do so. The most that can be hoped for is to indicate the broad outlines and applications of the technique and some of the results which have been obtained. For a general account of serology, the reader is referred to Boyd (1946), Topley and Wilson (1937), and Marrack (1938).

More specific discussions on the serology of viruses will be found in van Rooyen and Rhodes (1948) and Burnet, Keogh, and Lush (1937).

Immunity

There is more than one kind of immunity: the first is the innate or constitutional; the second is known as acquired immunity and is of two kinds, active and passive. Active immunity results either from recovery from infection or from artificial inoculation. Passive immunity is transmitted to an animal either naturally from the mother, or artificially by the injection of serum from an immune animal. Boyd arranges the different kinds of immunity as follows—

I. Innate immunity (constitutional or racial).
II. Acquired immunity.

(1) Active:

(*a*) Natural.
(*b*) Artificial.

(2) Passive:

(*a*) Natural (congenital).
(*b*) Artificial.

When animals are exposed to infection, there are produced in the blood and tissue fluids soluble substances which tend to prevent or cure infection. These are known as *antibodies* (because they are bodies acting *against* introduced substances). The substances which call forth the production of antibodies by the animal are called *antigens*

(because they *generate* the antibodies) and a substance which can act as an antigen is said to be *antigenic* (Boyd, 1946).

An antigen must have two properties: first it must have the power to stimulate the production of the antibody and secondly it must react specifically with that antibody.

There are four main types of this reaction between antigen and antibody—

(1) *Neutralization* of the properties of the virus;

(2) *Complement fixation test:* When antigens are mixed with their specific antibodies the mixture has the property of removing the power of normal serum to haemolyse sensitized red corpusles. *Complement* are a heat labile substance present in normal blood serum and the reaction is a kind of delicate colour indicator test;

(3) *Precipitin reaction:* A precipitate is formed when the virus is added to its specific antiserum in saline at different dilutions and warmed in a water-bath. In precipitation the antibody is referred to as *precipitin*;

(4) *Anaphylaxis:* *See* page 77.

The antiserum (or antibodies) to a given virus is produced by injecting a susceptible animal with a suspension of the virus in question. Rabbits can be used for some viruses and are easily immunized against vaccinia virus. The animal is inoculated sub-cutaneously with 0·25 c.c. of a 1 : 1000 dilution of vaccinia virus, because vaccinia is a skin parasite.

Similarly, antiserum to influenza virus can be produced in rabbits by the inoculation of two doses at weekly intervals of 5 c.c. of allantoic fluid from a culture of the virus in the developing hen's egg.

Guinea-pigs have also been used in the preparation of antisera to vaccinia, herpes, and psittacosis viruses. In the case of psittacosis, a mouse was first inoculated with the virus and its spleen removed and extracted with saline to make a 10 per cent suspension of tissue.

It must be remembered in preparing the viruses for inoculation into animals that many of the accompanying proteins from the tissues of the host are also antigenic and therefore the virus which is to be used for the production of antisera must be as free from contaminating tissue material as possible. Viruses cannot, like bacteria, be grown in pure culture on non-living, synthetic media, and this fact adds to the difficulty of preparing a pure virus suspension. It is easier with some animal viruses, than with others, to prepare a virus

suspension of high concentration; such are the viruses of vaccinia and the Shope papilloma which can be obtained in considerable quantities in the natural lesions.

The actual neutralization of the virus by its specific antibody is thought to be due to a reversible union between antibody molecules and certain determinant groups on the virus surface. When a certain amount of antibody has united, the virus particle becomes non-infective—the amount necessary may differ according to the method by which the infectivity of the particle was tested. If the concentration of virus is high enough, and sufficient time is given, collisions of the partially coated particles result in the formation of aggregates (Burnet, Keogh and Lush, 1937).

Chester (1936[a]) prepared neutralized mixtures of tobacco mosaic virus juice and immune serum by titrating the serum with the juice until the supernatant fluid after centrifuging contained an excess of neither serum nor virus, as determined by precipitin tests.

When such mixtures were subjected to a number of chemical, physical, and serological treatments, no free virus nor antibody was recovered, but when the mixtures were partially digested with pepsin, the antibodies were destroyed and a large portion of the virus was recovered, as determined by precipitin testing.

Tests for virus-neutralizing antibodies to animal viruses have been employed for the following purposes: (1) to assess the potency of therapeutic antisera with a view to their standardization; (2) for detecting the presence of virus-neutralizing antibodies in human or animal sera for diagnostic or epidemiological purposes; (3) to identify viruses or to investigate their antigenic structure.

The general principle on which these tests are based is the same: for the majority of instances the serum to be tested is mixed with the virus in suitable proportions, incubated for a short time, and inoculated into a suitable animal or an egg. In some tests, the amount of virus used is kept constant and different dilutions of serum are added; in others, the amount of serum is kept constant and the virus diluted instead (van Rooyen and Rhodes, 1948).

In considering the serology of plant viruses, we find an entirely different situation. Since, so far as we know, there is no antibody formation in plants, there is no acquired immunity to virus diseases in the accepted sense. The only type of acquired immunity in plants is of the non-sterile type. In other words, there is acquired immunity between related viruses only; thus, a plant infected with a tobacco

mosaic virus producing a green mottle cannot be infected with a related virus which gives rise to a yellow mottle.

The explanation of this type of immunity seems to be that a cell already fully parasitized by one virus cannot be invaded by another virus of a related type. It can, however, be infected by other and unrelated viruses. This phenomenon, incidentally, provides a useful method for identifying related viruses.

Nevertheless, plant viruses are potent antigens and when inoculated, in a fairly pure state, into a suitable animal, generally a rabbit, give rise to their specific antisera. Since this fact was first demonstrated in 1928 by Purdy (Beale) a great deal of intensive study on the serology of the plant viruses has been carried out.

In preparing antisera to plant viruses, the rabbit is the most suitable animal and the following is a brief description of the method. As already pointed out, it is important to have the virus sample as free as possible from plant proteins which are also antigens though not particularly good ones. This is usually done by precipitation with ammonium sulphate, followed by dialysis to remove excess of salt. (Chapter V on purification of viruses.) The following method is suitable for fairly pure virus preparations but for impure materials the interperitoneal injection should be used.

The rabbit used for preparing antisera should be large (about 4 lb) and should preferably have large ears with prominent veins. A 1-ml hypodermic syringe with a thin needle (about size 14) is used. The syringe is filled and air bubbles are expelled with the tip of the syringe held upright. The injection is made into the vein which runs along the upper surface of the ear, parallel to the hind edge and about $\frac{1}{8}$-in. to $\frac{1}{16}$-in. from it (Plate XVI, lower photograph). It is useless to try to use the other veins, even though they may appear larger.

If a rabbit is going to receive a series of injections, it is preferable to give the first near the tip of the ear and each later injection successively closer to the base.

The hair on the ear is smoothed down by wiping with cotton wool dipped in alcohol, or the ear may be shaved. The tip of the needle is inserted into the vein in the direction of the base of the ear, and 1 ml of the virus is injected. If the tip of the needle is in the vein, the liquid will flow in smoothly. The needle is withdrawn gently, slight pressure being maintained over the point of entry with the thumb of the left hand.

About two weeks after injection, the rabbit is bled from the other

ear. For this the ear is prepared by rubbing a very small amount of xylene or benzene on to its surface with a small wad of cotton wool. This is slightly irritant, causing the veins to fill with blood, and allows the bleeding to be done quickly and with a minimum of discomfort to the rabbit. A small cut is then made in the marginal vein near the base of the ear, using a small, very sharp instrument or a sharp piece of glass tubing. After sufficient blood has been taken, the flow can be stopped by applying slight pressure, and the cut sealed by a small amount of collodion dissolved in alcohol-ether.

The cuts for later bleedings are made successively nearer the tip of the ear. The blood is collected in a tube and left for some hours to clot. The serum is poured off and centrifuged to remove any remaining blood cells.

About 40 to 50 ml of blood can be taken on three successive days, followed by a further single bleeding at the end of a week. This gives about 100 ml of serum from one injection. The same rabbit after a rest period of a few weeks can then be used again.

Antiserum prepared in this way is not sterile and will deteriorate unless stored under conditions which prevent bacterial growth. This may be prevented either by keeping the material frozen or by the addition of a few drops of chloroform.

In either case it is advisable to keep the serum as cold as possible (Markham, Matthews, and Smith, 1948).

The fact that a plant virus will react with its own antiserum and with no other, by forming a precipitate when it comes in contact with it, is of great use in identifying a virus quickly without the necessity of making inoculation tests to "indicator" plants, and then having to await the results. The precipitin test is now much used in a practical manner to identify viruses, especially in the testing of potato plants, where it is important that the plants should be free of virus (Chester, 1937). The following considerations apply particularly to the large-scale testing of potato plants, grown for seed, for the presence of virus X.

One rabbit from one series of bleedings can give about 100 ml of antiserum. If 0·5-ml quantities of a dilution of 1 in 50 of the antiserum are used, the amount of antiserum is sufficient for 10,000 single plant tests. If groups of 10 plants were tested, 100 ml of antiserum would be sufficient to test 100,000 plants.

There are certain strains of potato virus X which give practically no symptoms on the usual test plants and could easily be missed in

inoculation tests. The serological test can pick up this type of strain just as easily as any other.

The facilities required for serological testing are not so extensive as for plant inoculation methods, the chief requirements being a suitable water bath to run at 50°C, a small power-driven centrifuge to take eight to twelve 15-ml tubes, and a supply of small tubes and pipettes.

The carrying out of serological testing is not limited by the seasons as the growing of test plants may be. The result of the test is known within an hour or so, compared with two to three weeks for the inoculation methods.

The precipitin test therefore is a useful tool for the identification of a specific virus but is not sufficient to distinguish between virus strains since all strains of the same virus will react with an antiserum prepared against one strain.

This merely indicates that the strains possess common antigens; it does not mean that they are antigenically identical.

Strains of the same virus can, however, be differentiated antigenically by the method of cross-absorption. This has been clearly explained by Bawden (1943) as follows—

> If each virus is not a simple unit antigen, but carries a number of different determinant groups, then the antiserum will also contain a number of different antibodies, each reacting specifically with its particular determinant group. The viruses which have one or more determinant groups in common will be precipitated equally by each other's antiserum. But if, in addition to the common antigenic group, each strain contains specific groups, then their effects on their homologous and heterologous antisera will be different. Each strain will react fully with its homologous antiserum, removing all the antibodies from it. It will remove from its heterologous antiserum, however, only those antibodies for which it has determinant groups, the others specific to the groups peculiar to the second strain being unaffected. The presence of such specific antibodies is shown by the formation of a precipitate when the virus strain used for producing the serum is added to a sample that has been allowed to react fully with a second strain.

Chester (1936[b]) carried out a separation and analysis of ten tobacco mosaic virus strains by means of cross-absorption tests. His results are given in Table V.

From this it may be seen that, after tobacco mosaic virus immune serum is allowed to react fully with aucuba mosaic virus extract, the serum is still capable of reacting with tobacco mosaic virus. The absorption of tobacco mosaic virus serum by an excess of aucuba

TABLE V

PRECIPITIN ABSORPTION TESTS FOR DIFFERENTIATING STRAINS OF TOBACCO MOSAIC VIRUS

Serum immune from:	Absorbed with extract of:	No. volumes absorbent required per volume of serum	Absorption precipitate	Residual precipitin reactions when tested against extract of:									
				Tobacco mosaic	Aucuba mosaic	Masked tobacco mosaic	J-102	J-108	J-201	J-202	J-302	J-303	J-306
1. Tobacco mosaic.	Aucuba mosaic	3	++++	++	0	++	+	0	+	+	0	0	+
2. Tobacco mosaic.	Masked tobacco mosaic	2	+++	0	0	0	0	0	0	0	0	0	0
3. Aucuba mosaic .	Masked tobacco mosaic	⅓	++	0	+	0	0	+	+	0	+	+	0
4. Aucuba mosaic .	Tobacco mosaic	½	++	0	+	0	0	+	+	0	+	+	0
5. Masked tobacco mosaic . .	Aucuba mosaic	2	++++	++++	0	++++	+	(+)	+	+	(+)	0	0
6. Masked tobacco mosaic . .	Tobacco mosaic	½	+++	0	0	0	0	0	0	0	0	0	0

(*After* Chester, 1936[b])

++++ = very strong reaction
+++ = strong reaction
++ = moderate reaction
+ = reaction safely positive but not strong
(+) = reaction weak and questionable.

mosaic virus does not remove all the antibodies against tobacco mosaic virus. In other words, tobacco mosaic virus contains antigenic material that is lacking in aucuba mosaic virus. Similarly, the tests with aucuba mosaic virus serum indicate that aucuba mosaic virus contains antigenic material that is absent from tobacco mosaic virus. The absorption precipitates show, however, that the extracts of these two viruses also contain common antigenic material. Chester suggests that the antigenic constitution of tobacco mosaic virus might be expressed as XY, that of aucuba mosaic virus as XZ, X being the common antigenic fraction, Y the fraction peculiar to tobacco mosaic virus, and Z the fraction peculiar to aucuba mosaic virus.

Serological methods can also be used to show that apparently unrelated plant viruses may contain common antigens and so are related strains. A good case in point is the relationship shown to exist between tobacco mosaic virus and two virus strains affecting cucumber known as cucumber viruses 3 and 4. This relationship could not be shown in any other way since the respective viruses have no common host plant on which cross-immunity tests might be made (Bawden and Pirie, 1937).

Conversely, serological methods will sometimes demonstrate that what was thought to be a single virus entity is actually several unrelated viruses. This is true of the tobacco necrosis viruses which are indistinguishable by biological methods (Bawden, 1941).

It has been previously pointed out that, as a general rule, a plant already infected with a given virus cannot be re-infected with a closely related strain of the same virus. With some plant viruses, however, where a large number of strains exist, this rule does not hold good with the more distantly related viruses and the cross-immunity test breaks down. It has recently been shown (Matthews, 1949) that out of a number of strains of potato virus X, some would cross-immunize against each other and some would not. The antigenic relationship of the pair of viruses that would cross-immunize was closer than that of the pair which would not, so that a correlation appears to exist between immunizing power and antigenic structure of the viruses in question.

Anaphylaxis

In general, when a guinea-pig is injected with an antigen (the anaphylactogen), if a period of ten or more days is allowed to elapse (the period of incubation), a state of sensitivity to the antigen develops

in this animal. When the same antigen is injected intravenously into an animal thus sensitized, a severe or fatal shock (anaphylactic shock) usually results. This anaphylactic reaction is so highly specific and can be induced by such small amounts of antigen that it is often utilized for the identification of substances where qualitative chemical methods are difficult (Beale and Seegal, 1941). This method was investigated by Chester (1936[c]) who used the Schultz-Dale technique. This technique is based on the fact that if a virgin female guinea-pig is properly sensitized with a protein, its excised uterine horns, when placed in an isotonic bath, will exhibit contraction when the specific protein used in sensitation is added to the bath. Such contraction can be conveniently recorded on a kynograph drum. Chester applied this method to several plant viruses including that of tobacco mosaic, but found that none of the viruses tested gave anaphylactic reactions. On the other hand, proteins from healthy tobacco and other plants were highly anaphylactogenic. By this means, then, it was possible to show that apparently pure preparations of tobacco mosaic virus protein actually contained a quantity of normal plant proteins.

The serological reactions can also be used as a quantitative measure of plant viruses. There are two methods, the precipitation end-point and the optimal precipitation point.

Beale (1934) first showed that the precipitation end-point method could be used for estimating virus concentration. The antigen she used was tobacco mosaic virus and she diluted the antigens until a point was reached at which a visible precipitate was just visible when mixed with antiserum. A quantitative relation was shown to exist between the antigenic content and the active virus concentration of eleven separate extracts compared in eight different pairs.

Beale determined the antigenic content by titrating the virus against a constant amount of antiserum and checked the infectivity by a biological method of plant inoculation. In the optimal precipitation point method the antigen is used at a constant dilution and the concentration of the antiserum varied. Bawden (1935) used this method in determining the virus content of various samples of the potato X virus. He used a semi-purified virus, one from which most of the plant proteins had been removed. The flocculation experiments were carried out in 7 mm-thick walled glass tubes, 0·9 c.c. of antigen at constant dilution being added to a series of tubes, each containing 0·9 c.c. of antiserum at varying dilutions ranging from $\frac{1}{4}$ to $\frac{1}{512}$. The tubes were then placed immediately in a water-bath at 50°C so that

the convection currents kept the tubes agitated. The bath was illuminated from behind in order that the tubes could be continuously observed. The optimal tube was taken as that which first showed any visible flocculation and the amount of serum is directly proportional to the amount of antigen. Bawden found that a close relationship existed between the antigen content, as measured by the optimal flocculation point with antisera, and virus content as measured by an inoculation method.

CHAPTER IX

CONTROL OF VIRUS DISEASES

In this brief review of the methods available for the control of virus diseases it will be easier to deal with the virus diseases of animals and plants separately. This is because the techniques are of necessity rather different, so much of the prevention and control of animal virus diseases being based on the development of an acquired immunity of a kind not existing, so far as we know, in plants.

Virus Diseases of Animals

The methods for the control of animal virus diseases are briefly discussed under two heads. In the one case the various types of inoculations are dealt with, together with other measures applied to the host itself. In the other case, possible methods of destroying the vectors of viruses are considered and in this process the new insecticides may play an important part.

1. Measures Applied to the Host

Under the general head of *vaccination* there are several techniques which are briefly discussed. Vaccination can be performed (*a*) with inactivated virus, (*b*) with active virus, (*c*) with modified virus, (*d*) with antiserum from a recovered animal, (*e*) with both active virus and antiserum, and (*f*) with combined active and inactive virus. It had for long been considered that inoculation with inactivated virus failed to produce immunity but now there is evidence that such inoculation is efficacious in some virus diseases. Probably one of the reasons for earlier failures was the use of inadequate amounts of inactive virus or the destruction of the antigenicity of the virus. We have learned already that it is possible to inactivate some viruses without altering their serological properties and this can be done by two main methods: (1) inactivation by physical agents, such as ultra-violet light, or visible light in the presence of methylene blue; (2) inactivation by chemical agents such as formaldehyde, phenol, or chloroform.

Although the application of inactivated virus vaccines is limited, some success has been obtained with dog distemper and equine encephalomyelitis. The use of formaldehyde for producing the

vaccines for dog distemper and equine encephalomyelitis has been found most satisfactory, but for fowl plague phenol is superior to formaldehyde.

A vaccine prepared from inactivated rickettsiae has been used for a number of years against the Rocky Mountain spotted fever. The vaccine is prepared from the infected ticks which are the vectors of the disease and each ml of vaccine contains the inactivated virus from $1\frac{1}{4}$ ticks. The ticks are raised in vast numbers, and infected adult ticks can be stored satisfactorily under refrigeration for at least $1\frac{1}{2}$ years before being used for vaccine manufacture.

It is interesting to find that an increasing amount of potent vaccine was due to the use of old infective ticks, since the virus in aged ticks seems to have better antigenic properties (Parker, 1941).

The use of vaccines containing active virus has had success with some virus diseases, such as fowl-pox, laryngo-tracheitis of chicken, and ovine ecthyma of sheep and goats. In these cases immunity is obtained by the administration of the active virus by unnatural or non-infective routes. Some viruses are infective only if introduced into the body by some particular portal of entry. Thus influenza virus must gain access to the respiratory tract in order to initiate the disease. If the virus is inoculated subcutaneously or intraperitoneally into ferrets or mice, no sign of illness follows, but a considerable degree of resistance against subsequent infection is established. Depending upon this, American workers have practised human vaccination with active virus given subcutaneously (Wilson Smith, 1939).

The greatest success by far, however, has been obtained by the use of modified virus vaccines, the classical case of this technique being vaccination against smallpox carried out by Jenner, a hundred years before the discovery of the first virus. He used the virus of vaccinia, obtained from calves suffering from cowpox, which is a mutant or modified form of the smallpox virus and is non-virulent, remaining localized round the site of inoculation. It nevertheless stimulates the production of antibodies effective against the more virulent and invasive virus of smallpox.

By different techniques, mutants of some viruses can now be produced artificially. There are two main methods: (1) by propagation of the virus in animals which are not the natural hosts, and (2) by culturing the virus on the chorioallantoic membrane of the developing hen's egg.

Perhaps the most striking instance of vaccination with a modified

virus is that of immunization against yellow fever. It was first shown by Theiler in 1930 that the yellow fever virus could be altered by passage of mouse brains and this was followed up by the further modification in chick embryo tissue culture. Eventually, a strain of yellow fever virus, known as 17 D, was evolved which was satisfactory for human inoculation and this has been much used. Indeed, during the year ending June, 1943, the Rockefeller Foundation distributed about seven and a half million doses of this vaccine.

Another virus disease which can now be treated with a modified but active vaccine is the mosquito-borne dengue or "break-bone" fever. Large-scale experiments were carried out in the U.S.A. during the late war and although great difficulty was encountered in finding a susceptible animal, the virus was eventually transmitted to a particular strain of white mice. This was only achieved after the virus had been concentrated by means of the ultracentrifuge. After repeated passage of many generations of mice, a strain of virus was evolved which produced only the skin rash, which is characteristic of dengue, without the fever and other symptoms. This strain was effective, however, in stimulating the formation of antibodies against the severe form of the disease as well.

The artificial modification of influenza virus to produce a vaccine is one of the most striking developments in the control of virus diseases. It began with the work of Burnet (1936) who found that a strain of the virus, propagated in ferrets, would multiply on the hen's egg membrane but gave no visible disease in the embryo. That it was multiplying could be shown by inoculation back to ferrets. However, with continued passage through eggs the virus began to produce visible pocks or lesions in the egg membrane. More passages led to an increase of virulence and the virus commenced to invade the chick embryo. Simultaneously with this increase of virulence for the chick embryo, there occurred a decrease in virulence for ferrets, mice, and men. The production of this egg culture type of influenza vaccine was carried out on a large scale in the U.S.A. during the late war to meet a possible recurrence of an influenza pandemic such as occurred in 1919 after the first world war. Two methods of preparing the vaccine were used. In one the virus was purified and concentrated by precipitation with chicken red blood cells. It was then removed from the blood cells by raising the temperature and low speed centrifugation to sediment the blood cells. The virus was then inactivated by formaldehyde.

In the second method, the virus-containing fluids were removed from the eggs, formaldehyde was added and the whole spun in a Sharples supercentrifuge. The sedimented virus was then re-suspended in salt solution and administered by sub-cutaneous injection about $\frac{3}{10}$ of a milligram of virus constituting a dose. This represents about half the material obtained from the fluids of a single chick embryo.

Since there is no cross-immunity between the two main strains of influenza virus, A and B, the vaccine to be effective must be a mixture of both strains of the virus.

As Wilson Smith (1939) has pointed out, the value of serum therapy in virus diseases is very limited and he suggests that the intra-cellular habitat of the parasite may largely explain why an antiserum is of little use once the disease has been established.

Since the serum of a recently recovered or artificially immunized animal contains antibodies which are capable of neutralizing the specific virus, serum-virus mixtures can be prepared which are completely non-infective. In some cases, mixtures are used where the amount of serum is insufficient to inactivate all the virus; a mild attack of the disease can then be induced which will give rise to a solid immunity.

Another method which has been used against cattle plague and dog distemper is to inoculate the virus on one side of the animal and then, after a brief interval to allow the virus to gain a foothold, inoculate the serum on the other side.

The story of inoculation against measles is an intriguing one and the following account is based largely on Lauffer's (1946) description of it. From studies on the composition of human blood carried out during the past twenty years, it has been discovered that the fluid component consists mainly of five proteins, albumin, fibrinogen, alpha-globulin, beta-globulin, and gamma-globulin. Furthermore, it was discovered that the antibodies to diseases are generally associated with the gamma-globulin fraction. The increasing use of blood transfusion, especially for the treatment of shock, led to the discovery that the constituent of blood most effective for the purpose is serum albumin. It is from these fractionating procedures that the inoculation treatment of measles finally arose. During the war, large stocks of human blood were accumulated, especially in the U.S.A., and the question naturally arose whether those fractions of the blood not needed for blood transfusion could not be put to some other useful purpose. Since such a large proportion of adult human beings are

immune to measles it was suggested that the gamma-globulin fraction of the processed human blood might be used as a means of controlling measles since it was sure to be rich in antibodies to the disease. In Boston, U.S.A., a number of experiments were carried out to test this possibility with very interesting results. It was found that if $\frac{1}{10}$ c.c. of the gamma-globulin rich fraction of processed blood was injected per pound of body weight, the individuals were completely protected. A quarter of this amount resulted in a very mild disease.

In New York City another experiment was carried out on 814 exposed individuals; 2 c.c. of gamma-globulin was administered uniformly to patients between six months and six years of age. Sixty-five individuals in a control group received no gamma-globulin. The results of this test were interesting: of the 814 patients treated none developed regular measles, 2 per cent had a moderate attack, 19 per cent had a mild form of the disease, and 79 per cent remained healthy. Of the sixty-five patients not treated, 48 per cent developed regular measles, 18 per cent moderate measles, 17 per cent mild measles, and only 17 per cent remained healthy.

It should perhaps be emphasized that where there is complete prevention of the disease, there is no subsequent protection, but modification to a mild attack confers long, if not life-long, immunity.

Since the serum of a convalescent, recovered, or artificially immunized animal contains antibodies which are capable of neutralizing the virus, it is possible to prepare serum-virus mixtures which are virtually non-infective. Such mixtures have been used to some extent against cattle plague and dog-distemper, the procedure usually being to inoculate the virus on one side of the animal, followed by an inoculation a little later on the other side with the immune serum.

Much greater success, however, has been obtained in immunizing dogs against distemper by a combination of active and inactivated virus and this success was due to the pioneer work of Dunkin and Laidlaw. The virus, inactivated by means of formaldehyde, is injected first, and this initiates a low-grade resistance which allows the later inoculation of active virus. By this means a solid immunity is built up.

In an experiment involving 650 hounds from twenty-three packs which had certainly been exposed to infection, the incidence of the disease was only 1·4 per cent and the death-rate only 0·3 per cent. Of a number of young hounds which were not inoculated almost all contracted the disease and half of them died.

Slaughter. In order to prevent the spread of a very infectious virus

of the lower animals and to eliminate a focus of infection, it is sometimes necessary to slaughter infected animals and their contacts. This policy of slaughter has been ruthlessly applied and rigorously carried out in the case of foot-and-mouth disease of cattle and pigs. It has been severely criticized from time to time, but there is no doubt that it has saved this country from much greater epidemics such as have been experienced on the continent. As soon as an outbreak of foot-and-mouth disease on a farm has been confirmed, the infected animals and any that have been in contact with them are slaughtered and the carcasses burned. The farm buildings are sprayed with disinfectant, any heavily contaminated material is burned, and the clothes of the farm workers are fumigated. At the same time, a "standstill" order on the movement of cattle into or out of the prohibited area comes into force.

The slaughter policy has recently been extended to the domestic fowl in an attempt to deal with another extremely infectious virus attacking fowls and turkeys. This disease is known as fowl-pest or Newcastle disease.

Another group of intensely infectious viruses are those attacking caterpillars and causing the so-called "polyhedral diseases." This type of disease is particularly destructive to silkworms and it has often been found more satisfactory to destroy the whole of the stocks of silkworms when the disease has become established and to start afresh rather than to try to clean up the stocks. Very careful disinfection is necessary, since the polyhedra are very resistant and retain their infective power for long periods.

Selective Breeding. There does not seem to have been very much selective breeding carried out in animals against virus diseases but Wilson Smith (1939) quotes the work of Webster (1937) in selective breeding of mice. By intensive in-breeding he produced four strains of mice:

(1) virus resistant, bacteria susceptible;
(2) virus resistant, bacteria resistant;
(3) virus susceptible, bacteria susceptible;
(4) virus susceptible, bacteria resistant.

Each of these types might be suitable for some problem in virus research.

Protection from Insect or other Vectors. Screening, or protecting in other ways, the susceptible animal from the vector of a virus is

applicable in a few cases. For example, horses can be kept free of African horse sickness by allowing them to graze in the open only during certain hours of daylight when the mosquito vector is quiescent and by stabling them at sundown in mosquito-proof buildings.

In certain parts of S. America the virus of a form of rabies is carried by "vampire" or blood-sucking bats, and here again the screening of windows and the protection of cattle is necessary.

Quarantine. Rigid quarantine regulations can be efficacious in preventing the entrance of a virus into a country. The almost complete exclusion of rabies from England by very strict regulations governing the import of dogs is a case in point. Again, foot-and-mouth disease is absent in Australia and this is apparently due to the embargo on the entrance of cattle from other countries.

Incidentally it is of interest in this connexion to notice the appearance in England of a new virus disease of dogs called *hard-pad*, because it is accompanied by thickening of the horny layer of the skin of the pads. It is transmissible to ferrets which also develop corns (MacIntyre *et al.*, 1948). This virus may possibly have entered this country in dogs smuggled in by allied soldiers during the late war.

2. Measures Directed against Virus Vectors

Attacking the arthropod vectors of a virus is not likely to lead to the complete control of a virus disease but may prove extremely successful if supplemented by other measures.

The classic case of this type of control measure was the attack on the mosquito in the Panama Canal zone. The early attempts to construct this canal had failed owing to the ravages of yellow fever, and it was only after the breeding grounds of the mosquito vector had been eliminated that success was achieved. During the trench warfare of the first World War, the louse-transmitted disease of trench fever caused great wastage of troops. Towards the end of the war, systematic attempts were made to keep the louse under control by steam sterilization of clothes and bedding.

The discovery of D.D.T. and other new insecticides now gives greater promise of success for this method of control. In 1944 an outbreak of typhus in Naples was stopped completely owing to the lethal effect of D.D.T. on the louse which transmits the disease. Because of the toxic action of this substance on mosquitoes and with the development of aerial distribution, some progress in the elimination of this insect vector may be expected.

The method of setting a thief to catch a thief or, to give it its proper name, *biological control*, may have some slight bearing on the control of virus vectors. The tick, *Dermacentor venustus*, transmits the rickettsial disease, Rocky Mountain spotted fever, and attempts have been made to reduce its numbers by breeding and liberating vast numbers of a Chalcid wasp which parasitizes it.

3. Elimination of Virus Reservoirs

The question of virus reservoirs is an important one and although little is known about them, there is good evidence that they exist for at least three virus diseases of animals.

These reservoirs of infection are usually other species which either react very slightly to the virus or do not react at all but still retain the virus in the blood. Such animals are known as *carriers*.

In the case of African horse sickness it is thought that some species of wild game may serve as a carrier. As regards jungle yellow fever, a careful survey was made of a large number of miscellaneous wild animals of the jungle to find out if any were susceptible to infection. More than two thousand wild animals were captured and inoculated and it was found that none of the animals were killed by the virus and few showed any sign of illness. Nevertheless many species, after inoculation, had virus circulating in the blood stream whilst the animals were running about. This is important because it would allow time for the mosquito to pick up the virus even if it circulated in the blood for only a few days. Once the mosquito itself was infected, however, it would retain the virus for the rest of its life which may be several months under favourable conditions. It is theoretically possible, therefore, for many types of animal to act as carriers of the virus of jungle yellow fever, but whether, during the above survey, any of the animals tested were naturally infected with the virus is not known.

Many attempts have been made to find a natural reservoir of the mosquito-borne virus of equine encephalomyelitis in the U.S.A., and there is some evidence that birds may be symptomless carriers. At the moment, the so-called "prairie chicken" seems to be implicated.

That a subject may be his own carrier of a virus is shown by the existence of the so-called latent viruses which rest quiescent in the body and are stimulated into action under circumstances which presumably lower the resistance in some way. *Herpes simplex* is a good example of this and presumably, also, the virus of the common cold.

4. Chemotherapy

The great success of chemotherapy against bacterial diseases raised hopes that similar results would be forthcoming against virus infections but, unfortunately, this is apparently not the case. Just recently, however, encouraging results have been achieved with two new antibiotics against certain virus diseases. It may be significant, however, that these antibiotics appear to react only with the very large viruses, the rickettsiae, and not with the small viruses. Whether this indicates that the mode of reaction of the very small viruses, like those of yellow fever, poliomyelitis and foot-and-mouth disease, differs fundamentally from that of bacteria and the rickettsiae remains to be seen.

Chloromycetin, the first of the two new antibiotics to be discovered, has been used with success against scrub-typhus and certain related infections. *Aureomycin* (trade name *Duomycin*) is the name given to the other antibiotic and it is a crystalline faintly golden yellow substance, a metabolic product of *Streptomyces aureofaciens*, a hitherto undescribed species of the actinomycetes. Although aureomycin has no apparent *in vitro* activity, it has marked therapeutic action against the viruses of psittacosis and the lymphogranuloma group and the rickettsiae of spotted fever, typhus fever, scrub-typhus fever and "Q" fever when grown on the chorio-allantoic membrane of the developing hen's egg, and in mice and guinea-pigs. It gave no results with the viruses of influenza B, dog distemper, and the MEF-1 strain of poliomyelitis virus.

Promising results were obtained in human patients suffering from Rocky Mountain spotted fever and fifteen "Q" fever patients were successfully treated by means of aureomycin, orally administered. An account of this work is given in a number of papers in the *Ann. New York Acad. Sci.*, 51 (1948), pp. 175–342.

Although the antibiotics tested so far against poliomyelitis have proved ineffective, some interesting results have been obtained in the chemotherapy of this virus in mice. It was found that pentnucleotide and yeast nucleic acid appeared to protect 90 per cent of mice exposed to the MM virus which produces a polio-like disease in these animals. Promising results on these lines have also been obtained with a human strain of polio virus in monkeys. In experiments in the Children's Hospital in Philadelphia it has been found that the drug, oxythiamin, which is an analogue of thiamin and nitrous acid, induced a resistance to poliomyelitis in mice by producing a deficiency of vitamin B_1.

5. Measures of General Hygiene

Included in this category are the obvious routine precautions of isolation and quarantine which reduce to a minimum the contact between the diseased and healthy subject. In the cases of influenza and the common cold it is hardly necessary to point out the danger of unrestrained sneezing and coughing. The droplets from a sneeze remain suspended in the air for more than ten minutes. A large handkerchief carefully used prevents the scattering of these droplets and a simple impermeable mask collects the vast majority of them. The use of the hand for checking such scattering is very inefficient (Bourdillon and Lidwell, 1941).

Apart from this more obvious method of air-borne infection of comparatively large droplets, attention is now being directed to the possible spread of these two viruses on dust and in a more or less dry condition. It has been demonstrated (Edward, 1941) that, after impregnation of a blanket with a suspension of influenza virus, the virus survives drying under ordinary atmospheric conditions and can be distributed in the air on dust particles by shaking the blanket. It was also found that the virus would withstand drying on a variety of other materials including household dust. The time for which the virus remained active was also estimated. There was little depreciation after 3 days, 10 per cent might persist for a week, and 1 per cent for a fortnight. Disappearance of the virus was much more rapid when impregnated material was kept at 37°C or in the light. Recent work of C. H. Andrewes and his collaborators on the common cold virus suggests that spread may take place through this virus being shaken out of handkerchiefs, especially when the latter have been used in the later stages of a cold. The possibility is suggested that impregnation of handkerchiefs with a disinfectant might make them less dangerous in this respect.

Some cheap and efficient method of destroying bacteria and virus particles in the air is badly needed. A sodium hypochlorite mist is fairly effective (Andrewes *et al.*, 1940) and ultra-violet light is useful under certain circumstances, but too expensive.

Virus Diseases of Plants

There are various methods for the control of plant virus diseases and there are two main avenues of approach to the problem. The first is to ward off, or avoid exposing the plant to, infection, and the second

consists of dealing with the plant itself in different ways. These methods are summarized under five heads.

1. Measures against Insect Vectors

As with the animal viruses, complete elimination of insect vectors is impracticable, though a certain amount of success in conjunction with other measures is possible.

There are various methods of approaching the problem, such as spraying or dusting, screening, fumigation, systemic plant poisons, avoiding the insect vector, biological control, and trap crops. So far, spraying to control the insect vector of plant viruses has had only a limited application, but with the discovery of new insecticides and the development of aerial dusting this method may yet prove effective. Screening the plants to keep off the insects has been used to some extent, mostly in the U.S.A. It was found that upright, low wooden frames, covered with cheese cloth or similar material were effective in warding off the leaf-hopper vector of the aster-yellows virus from the aster beds. In those parts of N. America where the aphis-borne viruses of cruciferous crops are of serious economic importance, the seedlings are covered with light canvas frames, until the plants pass the most susceptible stage for infection.

The attempt has also been made to grow seed potatoes under canvas frames to protect them from the several aphis-transmitted viruses.

Fumigation is mainly applicable to horticultural crops under glass where routine fumigation with nicotine will keep down the aphis and thrips which are the insects which transmit the virus diseases of horticultural crops in Great Britain. Nicotine fumigation of strawberry fields is also practised in this country and is carried out under a canvas sheet slowly towed over the plants, the nicotine vapour being pumped under the canvas by the towing vehicle.

There are certain chemicals which are taken up systemically by plants when watered into the soil and this offers a possible method of control of insects of the sap-sucking type such as aphides and leaf-hoppers.

By choosing the site or by timing the planting of crops it is sometimes possible to avoid the insect vectors of virus disease. Perhaps the best example of this on a large scale is the production of seed potatoes in those areas of Scotland where the climate is unsuitable for the aphis vector of potato viruses.

The biological control of insect vectors by the encouragement of their natural enemies is a possibility and a start has been made on the collection of parasites of the mealy bugs which transmit the swollen-shoot disease of cocoa.

Finally the method of trap crops may be mentioned; this is an attempt to draw off the insects from the main crop by early planting of a strip which is allowed to become infested and is then destroyed by chemicals.

2. Elimination of Reservoirs of Infection

This is a very important measure in the control of plant viruses because of the common occurrence of plant carriers and the wide host range of some viruses.

Cutting-out measures similar to the slaughter policy adopted with some animal viruses may occasionally be necessary when it becomes advisable to sacrifice a heavily infected crop.

The importance of starting with a virus-free crop cannot be over-emphasized since the presence of virus-infected plants in a young, growing crop, at the start of the season, offers ideal conditions for the spread of an insect-borne virus. This is particularly true of potatoes, and is the basis of the important trade in seed potatoes between England, Scotland, and Ireland. In the two latter countries, there are large areas with a damp climate unsuitable for the aphis vectors where virus-free seed potatoes can be raised on a scale not possible in England. It must be understood, of course, in this connexion that if a potato plant is infected with a virus then all the tubers from that plant, including the "seed" potatoes, are also virus-infected. It is this fact which makes virus diseases of such fundamental importance in all crops which are vegetatively propagated, including, besides potatoes, strawberries, raspberries, hops, and all bulbous and tuberous plants. For a comprehensive survey of the factors underlying the spread in the field of potato viruses the reader is referred to a recent monograph (Doncaster and Gregory, 1948).

The fact that so few viruses are transmitted through the seed makes it easy enough to start with a virus-free crop of sugar beet. But this crop is susceptible to two serious aphis-borne viruses, those of mosaic and yellows, and the question of sources of infection is a very important one to the grower. There seem to be three main sources, "volunteer" sugar beets left over from previous years, mangold clamps which may harbour both the aphis vector and the viruses, and thirdly the close

proximity of seed and mother beets which overwinter and are usually heavily infected with virus and aphis.

It is important, therefore, to separate the root crop and the mother-beet seed crop as far as possible and a scheme has been recently put forward for growing the "steckling" beets in another part of the country where sugar beets are not grown and where the likelihood of virus infection is less and then transporting them the following season to the sugar beet area.

It is a good general rule to avoid, whenever possible, the planting of a susceptible variety near to other varieties which are known to act as symptomless carriers of a virus. This applies particularly to raspberries where the well-known variety Lloyd George is a carrier of the mosaic virus; to strawberries of which Royal Sovereign is almost invariably virus-infected without showing symptoms, and to hops and dahlias.

3. Plant Breeding for Virus Resistance

Breeding new varieties of plants for resistance to virus infection is a hopeful line of attack and is one which has already had some success. The resistance may vary in kind and in degree but the end result is what matters. For example, much work has been done, both in this country and in the U.S.A., on the development of virus-resistant potato plants. The American potato known as 41956 is apparently completely immune to infection with the common potato virus X. On the other hand, potato varieties have been produced which are so susceptible to a given virus that they are killed outright. This is also a type of immunity since the virus is destroyed with its host; this kind of reaction is called *field immunity*. Sometimes a plant may be made resistant to a virus disease because it has been rendered unpalatable to the insect vector by reason of hairiness of the leaves or thickness of cuticle. Other outstanding examples of the production of virus-resistance in crops are the P.O.J. strains of mosaic-resistant sugar cane, the American varieties of sugar beet (U.S. Nos. 1, 33, and 34) which have a fair degree of resistance to curly-top and strains of cotton of Sakel type which are resistant to the leaf-curl disease.

Much of the success of this kind of breeding work depends upon the occurrence of a naturally immune or resistant individual plant so that a factor of resistance from it may be incorporated in a new line.

Again, it is possible to incorporate a character of resistance from one plant species in another by cross-breeding. An interesting example

of this is shown by some breeding work on the tobacco plant. The virus of tobacco mosaic, which causes a disease in tobacco of great economic importance, develops only local lesions without systemic spread in a related species, *Nicotiana glutinosa*.

The gene which thus localizes the virus has been transferred to the tobacco plant so that infection with the virus is confined to the point of contamination and the disease is unable to spread from plant to plant.

4. Treatment of Virus-diseased Plants

Once a plant has become infected with a virus, it is usually impossible to cure it, but there are one or two exceptions to this rule. In certain cases where the infecting virus has a low thermal inactivation point, a plant may be cured by subjecting it to heat treatment. Kunkel (1936) was able to cure peach trees, infected with the viruses of peach yellows, little peach, red suture, and rosette. The trees were kept at a temperature of about 35°C for a fortnight or more and the time necessary was longer for large trees than for small and it was easier to destroy the virus in the top of the tree than in the roots. That the virus was actually destroyed was demonstrated by grafting a scion from a cured tree on to a healthy one which remained healthy. Cured trees could be re-infected with the same virus, which shows that it was not a question of attenuation of the virus by the heat. It also demonstrates that there is no acquired immunity of the type we have discussed in the animal viruses.

Later, Kunkel (1941) showed that certain plants infected with the virus of aster yellows could be cured by similar means. It was only certain plants, however, not including the aster, which could survive being grown at 40°C for two weeks.

There seems to be only one case known of the chemotherapy of plant viruses. Stoddard (1942) states that he cured buds from peach trees affected with X-disease by soaking them in water solutions of quinhydrone, urea, and sodium thiosulphate.

5. Protective Inoculation

Since, so far as we know, there are no antibodies formed in plants, there is no vaccination in the manner applicable to the animal virus diseases. There is, however, one form of inoculation possible and it is mentioned here, though at the moment its interest is purely academic. We have seen in Chapter VIII how one virus will protect a plant from

invasion by a second virus provided the two viruses are closely related strains of the same type. Now if, as sometimes happens, one virus strain produces a very mild disease whilst the parent or type strain gives rise to a severe one, a plant can be first inoculated with the mild strain and thus be protected from infection with the severe strain.

Mild symptomless strains occur in tobacco mosaic virus and in potato virus X, and some preliminary attempts at protecting potatoes and tomatoes by this means have already been made.

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